2017

Defining and modulating the butyrate-producing microbial community in the swine gut

Julian Michael Trachsel
Iowa State University

Defining and modulating the butyrate-producing microbial community in the swine gut

by

Julian Michael Trachsel

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

Major: Microbiology

Program of Study Committee:
Heather K. Allen, Co-major Professor
Gwyn Beattie, Co-major Professor
Michael Wannemuehler
Nancy Cornick
Elizabeth Swanner

The student author and the program of study committee are solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred

Iowa State University

Ames, Iowa

2017

TABLE OF CONTENTS

CHAPTER 5: PROGRESS TOWARDS RESEARCH GOALS AND DIRECTIONS FOR FUTURE WORK

ACKNOWLEDGEMENTS

I would like to acknowledge and thank all the people that helped and supported me in completing my dissertation research. I sincerely thank my committee members Gwyn, Mike, Betsy, Nancy, and Heather for their kind support and critique of this research. Thanks to everyone at the NADC who put up with me in the lab, and helped along the way. Thanks to my partner and best friend Haley who was an amazing resource in making the figures contained in this document. A special thanks to my advisor and friend Heather Allen who gave me many fantastic opportunities and trusted me with immense freedom in the lab. Her support and guidance were a key part of my scientific training.

CHAPTER 1. CONTRIBUTIONS OF THE GUT MICROBIOTA TO HOST HEALTH AND RESISTANCE TO DISEASE

Summary

Swine are an important agricultural commodity, and global swine production is increasing. Currently antibiotics are extensively used in commercial swine production for the treatment and prevention of diseases. However, the rise of antimicrobial resistance (AMR) necessitates that we reexamine our use of antibiotics, and reduce their use whenever possible. Alternatives to antibiotics are needed if we are to successfully mitigate AMR while still ensuring the health of swine and the safety of the food they produce. The intestinal microbiota is increasingly being manipulated in a number of ways to provide alternatives to antibiotic treatment, and progress is being made in this regard. Here we examine the role of the swine intestinal microbiota, particularly the butyrate-producing community, in health and resistance to disease, and investigate how manipulations of this community can provide an alternative to antibiotics. What follows is a broad overview of the intestinal ecosystem, the beneficial functions performed by intestinal symbionts, some methods to manipulate these communities, and a summary of how the subsequent work outlined in this manuscript contributes to the goal of utilizing the functions of the gut microbiota as a way to promote health and reduce antibiotic treatment.

Introduction to the gut microbiota

Microbes are ubiquitous on our planet and are critical for understanding any biological system, particularly an organism's health and resistance to disease. Virtually every macroscopic organism has a robust community of microbial symbionts colonizing every exposed epithelial surface. These symbionts are important for the health and survival of their hosts, and in many cases the host and its symbionts are inseparable (1, 2). Mammals are not exempt from heavy microbial colonization, and every tissue contacting the outside environment is colonized by microbes (3). Different tissues have different microbial communities reflecting the conditions of each site. Often, these microbial communities are not passive hitchhikers, but have an important role in the functioning and health of these tissues. Different regions of skin have distinct microbial communities (4), and variation within these distinct communities have wide-ranging implications, from severity of underarm odors (5), to susceptibility to foot fungus (6). Similarly, the vaginal microbiome is highly specialized to exclude potential pathogens from colonizing these tissues (7), and deviations from a healthy community structure has serious health implications such as altering susceptibility to HIV infection (8). These different microbiomes are undeniably important; however, the microbiome of the gastrointestinal (GI) tract has a special role in the health of the host organism.

Two main GI tract conformations exist: foregut fermenters (ruminants, e.g., cattle, bison, deer) and hindgut fermenters (e.g., mice, humans, swine). The main difference between these two configurations is that foregut fermenters have a specialized organ, often called a rumen, which is a large microbial fermentation vat located after their esophagus at the beginning of the GI tract. In the rumen, a complex ecosystem of microbes digest plant matter and ferment it into short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate that host tissues absorb.

Because initial digestion is delegated to microbes, the host need not produce all the enzymes required to digest the diverse plant-derived substrates from the diet. Instead, microbial genomes encode these capabilities, and they encode a great diversity of enzymes to participate in the breakdown of dietary substrates. Additionally, as the microbial communities in the rumen change in response to new food substrates, so too does the enzymatic digestive capacity of the rumen (9, 10). Foregut fermenters are so dependent on their microbial partners that they cannot survive without them; this is in contrast to hindgut fermenters, such as humans and swine, where the host can technically survive without intestinal microbes. In hindgut fermenters the colon and cecum are somewhat analogous to the rumen of foregut fermenters, and the remainder of this chapter focuses on the role of the intestinal microbiota in health and disease of hindgut fermenters, with a focus on swine where data are available.

Importance of studying the swine gut microbiota

Much more effort has been expended researching the intestinal microbiota of humans and lab rats compared to swine; however, investigating swine-associated microbes and their interactions with the host is important. Humans and swine have remarkably similar GI tract configurations, and swine are generally considered to be much more similar to humans than the most popular laboratory animals used for biomedical testing: mice, rats, and dogs (11). Swine are omnivorous just as humans and have the same general gut segments, though these differ in some ways. The gross anatomy of the small intestine differs slightly in that swine have relatively longer sections of duodena and shorter ilea than humans, though the differences are more pronounced in the large intestine. Swine have a very pronounced cecum, which is the dead-end pouch directly after the ileum. This structure in humans is little more than an enlargement of the proximal colon just distal of the ileum (11). The anatomic layout of the swine

colon is quite different from humans. It is described as a 'spiral colon' being composed of several spiraling segments that loops in on itself as it progresses towards the rectum. Gross anatomy aside, the structure and function of the epithelia and protective mucus layer are quite similar between the two species. Researchers have successfully transplanted human microbes into the swine intestinal tract, generating swine with humanized gut microbiota (12), showing that the environments within the two species are similar enough to support the same microbial populations under artificial conditions.

In addition to the pig's similarity to humans and its use as a biomedical model organism, swine are a globally important food animal. Antibiotics are used frequently in swine agriculture and their use is expected to continue to rise (13). Consequently, swine have been identified as a potential source of multidrug-resistant bacteria as well as zoonotic enteric pathogens (14). Knowledge of the intestinal microbiota of swine is critical to help develop strategies to mitigate these concerns. The pig's use as a model for human health as well as its global importance as a livestock animal make understanding the intestinal microbiota and its interactions with the host of great interest to both livestock producers and medical professionals.

Differing niches throughout the GI tract

The GI tract consists of all the tissues spanning from the oral cavity to the rectum, and the distribution of microbes is different throughout the different gut sections. The epithelia of the GI tract represents the largest exposure of host tissues to the outside environment, and in humans the GI mucosa has a surface area of over 40 m^2 (15). Much of this area is specialized absorptive structures in the small intestine. Microbial populations are present throughout the entirety of the GI tract, and different gut locations have different populations of microbes reflecting the differing environments at each of these locations (Figure 1).

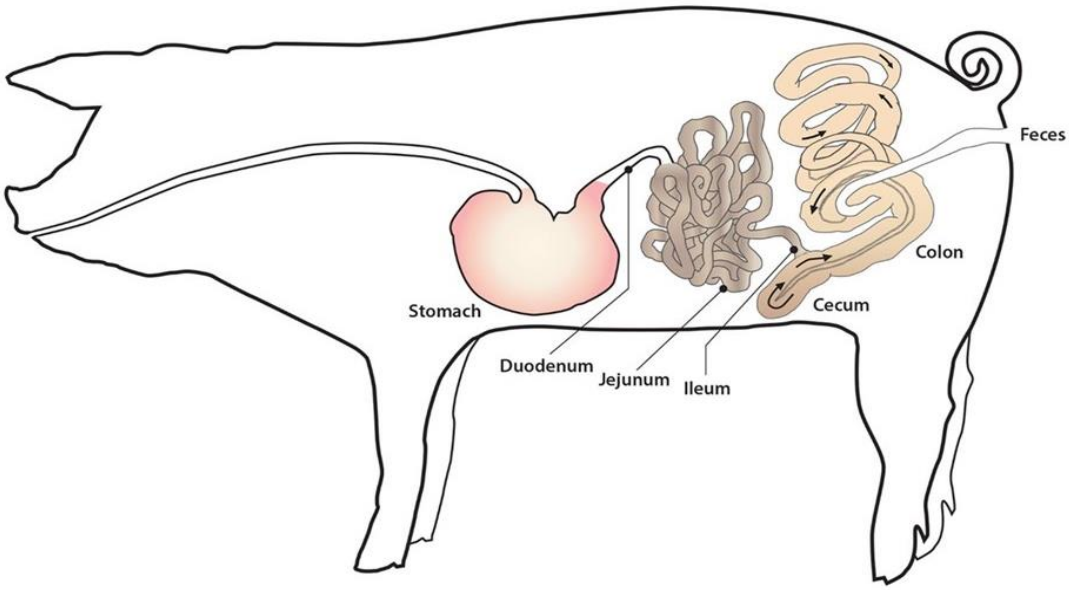

Figure 1: A Diagram of the swine gastrointestinal tract. Each gut segment is labeled and the arrows indicate the direction of digesta flow.

The microbial load increases from the oral cavity to the rectum (16). The oral cavity, esophagus, and stomach have relatively few viable microbes per gram contents, and the numbers of bacteria gradually increase through the small intestine. The cecum and colon have the highest

population densities, estimated be upwards of 10^{11} cells/gram contents, making it one of the densest microbial communities known in any environment on the planet (17). Many host factors also restrict the amount of microbial growth that occurs in gut segments more proximal than the distal ileum, and overgrowth of bacteria before the colon can have negative health consequences (18, 19).

Several factors impact the distribution of microbes through the GI tract. A pH gradient is present, with the pH being lowest in the stomach (pH = < 2), increasing through the small intestine where it reaches neutral or slightly basic pH in the distal ileum (20), and dropping again to between 5.5-7.0 in the cecum and colon. Similarly, an oxygen gradient exists throughout the GI tract. The stomach and its contents are highly oxygenated, with the partial pressure of oxygen being only slightly lower than atmospheric conditions (21). The lumen of the small intestine is considered to be aerobic, but in the distal small intestine the concentration of oxygen begins to fall. In the cecum and colon the luminal contents are anaerobic, with the partial pressure of oxygen being very low. Both host and microbial activities are responsible for this gradient. In addition to this longitudinal oxygen gradient, a radial oxygen gradient exists as well. This gradient is most pronounced in the cecum and colon. The mucosal tissues receive a constant supply of oxygen from the bloodstream so they remain oxygenated, and it has been demonstrated that this oxygen can diffuse into the lumen (22). Often facultative anaerobes colonize the mucosa and take advantage of the available oxygen to utilize respiratory metabolisms (23).

The host produces a mucus layer that covers nearly all epithelial surfaces in the GI tract, creating additional niches for microbial colonization. The mucus layer serves as the first barrier between the outside world and the intestinal mucosa and also helps to lubricate and prevent mechanical damage from the food bolus as it moves through the GI tract (24). This layer is

composed of individual mucin proteins that are made of a heavily glycosylated protein core. Mucins come in two distinct types: secretory and membrane-bound. Membrane-bound mucins are physically anchored to epithelial cells, while secretory mucins are secreted by specialized cells known as goblet cells (24). These two types of mucins work cooperatively to form a robust mucus barrier with the goal of protecting host tissues from environmental insults and invasion by commensal bacteria.

Two distinct mucus layers exist in the gut: a firmly adherent mucus layer directly adjacent to the epithelia, and a loosely adherent mucus layer located towards the lumen. Both of these layers are primarily composed of the MUC2 protein and contain many barrier-enhancing proteins such as host secreted antimicrobial peptides (AMPs) and secretory IgA (sIgA) (25, 26). In healthy animals, the firmly adherent mucus layer is nearly devoid of bacteria, but the loosely adherent layer is heavily colonized by diverse populations of microbes (27). The mucus layer is a rich source of carbohydrates for intestinal bacteria; as much as 70% of the weight of the MUC2 protein is composed of O-linked oligosaccharides, which are a good carbon source for bacteria. The loosely adherent layer is generated from the firmly adherent layer as commensal bacteria break down sugar linkages and the cores of the MUC2 proteins. Many different types of bacteria harvest host-derived glycans from the mucus layer (28), and this digestion of the mucus layer is normal and healthy so long as the rate of mucus degradation does not exceed the rate at which it is excreted (29). The communities of bacteria associated with the mucus layer often differ from those in the lumen, reflecting the differing carbon-source availability and differing redox potentials (30). The gradients and differences in environmental conditions throughout the gut create unique niches that are filled with a variety of different bacterial species.

Biogeography of the intestinal microbiota

The microbes that colonize each gut location reflect the environmental conditions at these locations. Several surveys of the biogeography (physical distribution species) of the intestinal microbiota have been conducted in a variety of animals, and similar trends are detected between different species. While the majority of available data is from the human gut microbiome, many swine-specific studies have been performed. Generally, different gut locations have different community compositions, but community compositions of gut locations also differ between the lumen and mucosa. The majority of species detected in the gut belong to 4 main phyla, *Firmicutes, Bacteroidetes, Proteobacteria,* and *Actinobacteria* (31), and many members of these phyla have specifically adapted to the host-associated gastrointestinal environment.

Very little diversity is detected in stomach contents, reflecting its harsh environmental conditions; however, the stomach is not sterile as once thought. Species belonging to the genera *Veillonella, Lactobacillus,* and *Clostridium* have been cultured from gastric juices (32), though they are thought to be transient members of the stomach microbiota (33). The gastric mucosa, however, has been shown to have more permanent residents. Bacteria belonging to the genera *Lactobacillus, Streptococcus, Veillonella, Prevotella,* and *Helicobacter* have been routinely detected in the gastric mucosa through traditional and culture-independent techniques. A meta-analysis of swine gut microbiota recently demonstrated that the *Lactobacillus* and *Acinetobacter* genera are common members of the swine gastric mucosa (34), and an additional study suggested that the gastric mucosa can harbor diversity comparable to other GI segments (35).

In the small intestine, the richness of the bacterial community begins to rise. Several studies of the swine gut microbiota have examined bacterial diversity in the small intestine, although very few samples are available for the segments more proximal than the ileum (34).

Generally, the microbes that inhabit the ileum belong to the phylum *Firmicutes*, although *Proteobacteria* are often represented as well (36). Notably, many studies have shown that the phylum *Bacteroidetes* is not abundant in the ileum, despite being a major phyla elsewhere. In swine, it has been demonstrated that the ileal mucosa harbors significantly more diversity than the luminal contents, and it has been postulated that this community may serve as a "seed" community for the cecum and colon (36). Metagenomic investigations of the ileal microbial community suggest that this community is not important for the breakdown of complex resistant dietary fibers, but rather takes advantage of small molecules such as mono- and disaccharides (36, 37).

Although the microbiota of the ileum may not be crucial for the processing of recalcitrant dietary substrates, it has been suggested to be a vital part of the education of the intestinal immune system. Swine (as well as humans) have specialized immune structures known as Peyer's patches throughout the small intestine, and these structures are particularly dense in the distal ileum (38). The Peyer's patch is a structure that is visible by the naked eye and appears as a rough, raised area of the intestinal epithelium (39). These patches are made up of a high density of microfold cells (M-cells). These are specialized structures that are designed to allow immune cells to sample antigen from the intestinal lumen (40). In swine, these structures have been shown to be important for the education of various types of immune cells, and antigen sampling in these structures can dictate the types of immune responses to colonizing commensal microbes (38). It follows that the microbes that colonize the ileum and ileal mucosa play an important role in the host immune response to commensal organisms.

The cecum and colon, also known as the distal gut, are where bacterial activity and community densities are highest. The cecum in swine is a large pouch where vigorous microbial

fermentation of dietary fiber occurs. The bacterial populations that inhabit the cecum are distinct from those that inhabit other sections of the GI tract; however, some overlap in species composition occurs. The most notable difference is the increase in the proportion of bacteria belonging to the phylum *Bacteroidetes* in the cecum. These are Gram-negative anaerobic bacteria that are known for their ability to degrade and ferment complex dietary substrates (41). In swine the vast majority of species belonging to this phyla belong to the genus *Prevotella*. The abundance of this genus is likely a direct result of the diets commonly given to commercial swine, which are high in carbohydrates (corn) and low in fats and proteins. Many studies have shown that this genus thrives in the hindguts of organisms that consume diets rich in plant carbohydrates (42, 43). In further support of this connection, multiple studies have shown that a diet rich in fats and proteins and low in plant-based carbohydrates selects against members of the genus *Prevotella* (43, 44).

Many other types of anaerobic bacteria are in high abundance in the distal gut. The phylum *Spirochaetes* is also more abundant in the swine cecum and colon; this is primarily due to the abundance of the genus *Treponema* that has been shown to be common in swine (34). Bacteria belonging to the families *Ruminococcaceae* and *Lachnospiraceae* similarly increase in the distal gut. Although all of the aforementioned bacterial groups are common members of the distal gut of swine, a recent meta-analysis of swine has shown that no species are always found in the colon or cecum of every pig (34). Rather, many diverse bacterial species can thrive in the anaerobic environments of the distal gut and feed off of dietary fibers and host-derived substrates. The communities that are present are a result of the dietary substrates available, and diet has been shown to be a powerful driver of the community structure of the hind gut (45).

Although all of the bacterial populations at all segments of the GI tract have impacts on gut health, the distal gut harbors by far the most microbial diversity and metabolic activity. The interactions between the host and microbiota in the distal gut are essential to host health; some even go so far as to call the microbial communities in the distal gut the 'forgotten organ' (46). Only recently are the functions and interactions of the distal gut microbiota being appreciated for the systemic effects they can have on the host (47). Much of the remainder of this document will focus specifically on the microbiota of this gut segment, their functions and activities, the interactions between bacterial species and with the host, and how this knowledge can be leveraged to benefit host health.

Functions of the gut microbiota

Dietary fiber digestion

In hindgut fermenting animals, the intestinal microbiota are not technically essential for the survival of the host. That said, a healthy microbiota performs many important functions, and disruptions, or dysbiosis, in the intestinal microbial community can have serious health implications. Germ-free animals of many different species appear normal by most standard metrics of health but are much more susceptible to many pathogens because they do not have a microbiota to competitively exclude pathogenic invasion (48). Germ-free animals are also not able to extract maximal energy from their diets (49). This is because although most of the easily digestible substrates are absorbed by the host in the small intestine, dietary fibers (recalcitrant or structural polysaccharides such as chitin, cellulose, pectin, and resistant starches) are typically fermented by bacteria in the large intestine (50). Much of the microbial activity in the distal gut is directed at the digestion of complex polysaccharides whether they be of host or dietary origin. The ability of the gut microbiota to change in response to changes in the diet allows a large

plasticity in the types of fibers that can be digested in the distal gut. In fact, it has been shown that gut bacteria can even gain the ability to breakdown dietary substrates from environmental bacteria via horizontal gene transfer (51). In the process of feeding on dietary fibers, bacteria produce many metabolites and compounds, some that are absorbed by the host, and others that have impacts on other microbial symbionts.

Vitamin synthesis

Many bacterial groups synthesize several different vitamins that are absorbed by the host epithelia. B-group vitamins such as folate and riboflavin are synthesized by well-known probiotic strains of *Lactobacillus* spp. and *Bifidobacterium* spp. (52). A recent study suggested that the metabolic pathways for B vitamin synthesis are common in human gut metagenomes (53). Similarly, a complementary study of the genomes of common gut bacteria suggested that the genes for B-vitamin synthesis are widespread and are found among all phyla (54). Another study has suggested that the host has dedicated systems to absorb microbially produced B-vitamins in the colon (55), and it has been estimated that up to a quarter of the recommended daily intake of several B vitamins can be satisfied by microbial production in the colon alone (56). Similarly, several studies have also shown that vitamin K production by the distal-gut microbiota is important and has impacts on host health. Germ-free rats that were not given dietary vitamin K did not have adequate prothrombin levels (an important clotting factor) and had clotting disorders, while conventional rats (those with a gut microbiota) did not suffer the same adverse consequences of dietary deficiency of vitamin K (57). Similarly, humans with low dietary intake of vitamin K did not suffer deficiencies unless they were treated with broad-spectrum antibiotics, which depleted their intestinal microbiota (58).

Microbial alteration of dietary compounds

In some cases, bacteria in the distal gut do not directly create physiologically relevant compounds, but rather alter the compounds and therefore alter their effects on the host as well as the microbial ecosystem. One example of this is bile acid modification. Bile acids are released by the host to aid in the solubilization and absorption of fats. The majority of these are re-absorbed in the distal ileum, but a small amount reach the distal gut where microbes alter their structures and activities in a variety of ways. Bile has important signaling functions in many host tissues, and microbial alterations of bile acids alter their signaling capabilities and therefore affect the bile-associated signaling networks in host tissues (59). Additionally, bile has antimicrobial effects due in part to its ability to act as a detergent that can disrupt microbial membranes; however, these effects can be enhanced after alteration of their molecular structures (60). Many beneficial gut bacteria have intrinsic resistance to the antimicrobial effects of bile and this resistance is commonly used to identify potentially beneficial probiotic strains (61).

Plant polyphenols, also known as phytochemicals, are an area of active research due to their potential associations with health (62). Although this is a very diverse group of compounds, they often share the characteristic of having poor bioavailability, meaning they are poorly absorbed by the host in the small intestine and therefore reach the colon. Once in the colon, various microbial activities have been shown to alter the structures of these compounds (56). Once altered, these compounds are more readily absorbed by host tissues and can then perform their beneficial actions such as anti-inflammatory activity (63), anti-tumor activity (64), or anti-diabetic activity (65). Currently compounds in this category are being investigated to improve various aspects of animal production, including swine (66)

Education of the immune system

An additional important function of the gut microbiota is the education of the host-immune system. "Educating" the immune systems is the training of immune cells in regards to which antigens are foreign and which are self, and how to react to these antigens. The intestinal mucosa is the largest region exposed to the outside environment in a mammal based on surface area. Therefore, up to 70% of all immune cells reside in gut associated tissues, depending on the host species (39). Disturbances or dysbiosis in the intestinal microbiota have potential associations with many auto-immune diseases including allergies, asthma (67), type 1 diabetes, multiple sclerosis, rheumatoid arthritis, lupus (68), and other inflammatory disorders (69). The mechanisms and connections between dysbiosis and these diseases are still being clarified but current data suggest that the activities of the intestinal microbiota have important consequences for immune responses in the gut and in the body.

Microbial production of short chain fatty acids (SCFAs)

The previously mentioned functions of the gut microbiota are certainly important; however, the production of SCFAs by microbial fermentation is arguably one of the most important. Most SCFAs are produced by anaerobic fermentation of dietary fiber in the hindgut. Some fermentation occurs in the small intestine, but the low microbial biomass and the availability of oxygen for respiration limit the amount of SCFAs produced in these gut locations (70). Aerobic respiration also occurs in the distal gut but is a much smaller fraction of the total microbial metabolic process than anaerobic fermentation (71). The main SCFAs produced in the hindgut are acetate, propionate, and butyrate, generally occurring in ratios ranging from 75:15:10 to 40:40:20 (72). Other SCFAs are produced as well, but they are either produced in small quantities (valerate, caproate), or are metabolic intermediates (lactate, succinate) that are quickly

converted to other SCFAs (71). In the colon, SCFA concentrations can reach as high as 200 mM, although it can be difficult to accurately quantify the total production of SCFAs due to the absorption of these compounds by host tissues (73).

All SCFAs can be absorbed by host tissues and are utilized in a variety of ways. The colonic epithelia, as well as other cell types, can express several monocarboxylate transporter (MCT) proteins, and these are used to import microbial fermentation products from the lumen (74). Various MCT isotypes exist and all known varieties are symporters, which couple the transport of SCFA anions to the simultaneous transport of cations such as H^+ and Na^+ (75). SCFAs can also enter host tissues via passive diffusion, although this is of less importance than active transport (75). Once SCFAs have been absorbed, they affect the host in a variety of ways. The three main SCFAs (acetate, propionate, and butyrate) can be detected in the portal blood in much higher concentrations than peripheral circulation due in large part to extensive processing of these compounds by the liver. Acetate is oxidized in the tricarboxylic acid (TCA) cycle as an energy source, but is also used as a biosynthetic building block in the production of fats and lipids (49). Propionate is largely used for gluconeogenesis in the liver (75) although it has been shown that some immune cells use this fatty acid as a fuel as well (76). Butyrate that reaches the liver is mainly oxidized in the TCA cycle much like acetate. However, this fatty acid's true value is realized not in systemic circulation, but very near to the site it is produced: the intestinal mucosa.

Butyrate as a central regulator of intestinal homeostasis

Butyrate, a four-carbon SCFA, has many well-documented effects on intestinal health. As with other SCFAs, butyrate is utilized by the host as an energy source; however, unlike the other common SCFAs, butyrate is the preferred energy source for colonocytes (77) and is rapidly

absorbed and oxidized by the colonic epithelium. Beyond its use as a fuel source, butyrate is also able to induce transcriptional changes in the host epithelium. These changes in expression generally re-enforce the innate colonic defense barrier. It has been shown to induce expression of antimicrobial peptides and increase their levels in the colonic mucus layer (78). Similarly, butyrate stimulates the production of the MUC2 protein, increasing the secretion and thickness of the mucus layer. Administration of 0.1mM butyrate to human colon biopsies *ex vivo* was able to stimulate mucus production (79). Tight junction proteins are similarly affected, with butyrate causing an increase in their expression and a decrease in the permeability of the colonic epithelia (80). These effects together decrease the likelihood that intestinal microbes will contact host immune cells and elicit a response.

In addition to its ability to reinforce the colonic epithelial barrier, butyrate exerts potent immunomodulatory actions, most of which result in anti-inflammatory effects. Butyrate has been shown to reduce colonic inflammation by limiting the activity of pro-inflammatory CD4+ T cells. Additionally, the same study showed that butyrate also reduced the sensitivity of colonic epithelial cells to IFN-γ, a proinflammatory cytokine (81). Butyrate was recently shown to induce the expression of extra-thymic T-regulatory cells. These are immune cells that are involved in promoting tolerance of the commensal microbiota and generally dampen inflammatory processes in the gut (82). Similarly, butyrate has been shown to exert anti-inflammatory effects on macrophages from the lamina propria. Macrophages are one of the most abundant cell types in the intestinal lamina propria, and exposure of these cells to butyrate significantly down regulated their expression of LPS-induced pro-inflammatory mediators, such as IL-6 and IL-12 (83). While maintenance of immune tolerance is complex and requires a balance among many regulatory factors, it appears that butyrate is a signal for the host immune

system to inhibit pro-inflammatory responses and tolerate the populations of microbes that are present (84).

The role of butyrate in colonization resistance

Because of its potent immunomodulatory effects and its role in supporting the physical colonic epithelial barrier, butyrate plays a key role in the development of colonization resistance to gut pathogens. Initially it was thought that butyrate and other SCFAs provided colonization resistance through pH effects and pH-dependent antimicrobial action (85). Indeed, increased levels of SCFAs have been shown to inhibit the growth of members of the *Proteobacteria* phylum and other pathogens in vitro (86, 87). However, recently it has been proposed that SCFAs and butyrate in particular contribute greatly to colonization resistance by limiting the availability of terminal electron acceptors available for microbial respiration.

Products of the host immune response are one source of compounds that feed microbial respiration. When the intestinal immune system mounts an inflammatory response, it releases reactive oxygen species such as NO and peroxides. These in turn cause the generation of NO_3 and tetrathionate, which can serve as terminal electron acceptors in many different species. The pathogen *Salmonella enterica* perfectly demonstrates this phenomenon as it has been shown to exhibit chemotaxis toward host-generated electron acceptors (88), and *Salmonella* spp. genomes encode metabolic networks that enable increased growth in the inflamed gut (89). Butyrate, a powerful signal for immune tolerance, reduces inflammatory signaling and the production of immune-derived reactive oxygen species (90).

In addition, it has been shown that the consumption of butyrate by host tissues plays a central role in the reduction in mucosal availability of oxygen. Colonocytes pump water from

the lumen of the colon requiring a large amount of ATP (91). This ATP demand is satisfied via the oxidation of butyrate in the TCA cycle, a process which consumes large amounts of oxygen. Under normal conditions, so much oxygen is consumed that the colonic epithelia is strongly hypoxic. This limits the diffusion of molecular oxygen from the tissues into the mucus layer (92). It has been demonstrated in mice that depleting the butyrate-producing microbiota with antibiotics causes an increase in oxygen concentrations of the colonic mucosa. This leaves these mice susceptible to colonization by mucosal pathogens; however, colonization resistance can be restored via oral administration of butyrate (93).

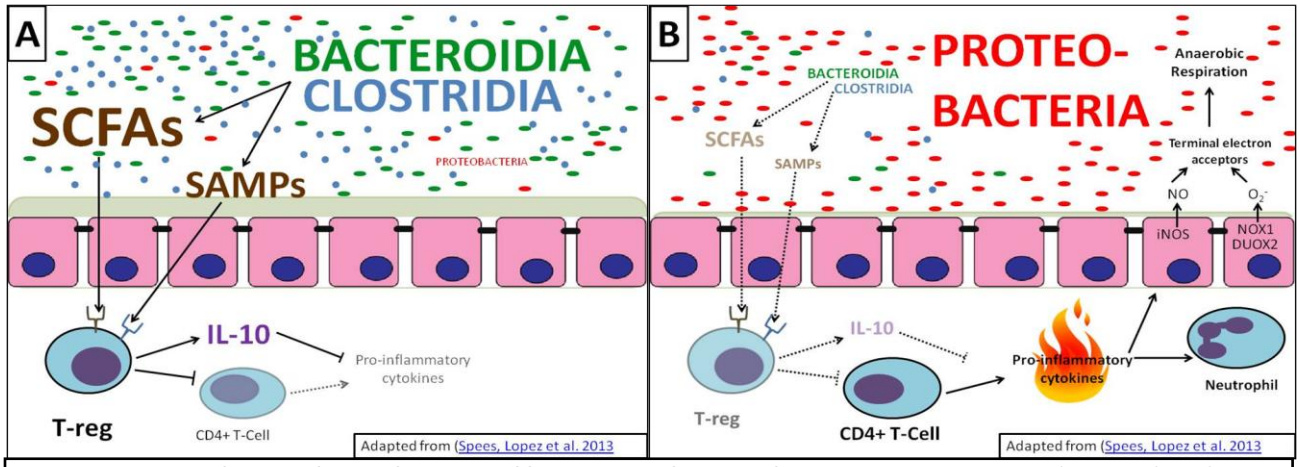

Figure 2: Interactions between host and commensal bacteria contribute to colonization resistance. A: Under normal conditions, interactions between commensal bacteria and the host, mediated by SCFAs and symbiont associated molecular patterns (SAMPs), establish conditions in which invasive members of the *Proteobacteria* phylum cannot compete and are present only at low levels. **B:** Any disturbance that causes alterations in the ratios of commensal bacteria can lead to reduced SCFA production, (including butyrate) causing increased levels of inflammation and oxygenation of the mucosa. Under these conditions, members of the *Proteobacteria* phylum can begin to utilize respiration and outcompete commensal bacteria.

Under normal colonic conditions, anaerobes can outcompete members of the *Proteobacteria* phylum or other groups capable of respiration, but with the appearance of terminal electron acceptors the balance is shifted and the invaders can outcompete the normal commensals (90) (Figure 2A and 2B). Many studies have shown that disturbances (reductions in normal commensal bacteria) in the gut are associated with, or followed by, a bloom in *Proteobacteria* species (94-98). These disturbances are detrimental to the normal function of the

distal gut and host health. Microbes that produce SCFAs (butyrate in particular) help to maintain a colonic environment that excludes pathogens by promoting immunological tolerance and maintaining hypoxic conditions in the epithelia, depriving pathogens or other invasive microbes of the ability to utilize respiratory metabolisms.

Manipulating the intestinal microbiota to improve host health and resistance to disease

Probiotics

Probiotics are live organisms administered to a host with the intent of beneficially manipulating the functions or composition of the gut microbiota. Currently many probiotics are commercially available yet, our understanding of manipulating the intestinal ecosystem with live organisms is not well developed (99). Perhaps due to our limited understanding of how these administered organisms integrate into the existing intestinal ecosystem, individual responses to the same probiotic treatments can vary considerably. One individual may experience significant benefits from a probiotic administration, whereas another individual receiving the same treatment may experience nothing (100-102). This phenomenon is likely due to differences in the established microbial communities of each individual, which vary significantly even among healthy individuals (3, 34, 103). It may be necessary to survey the existing community prior to probiotic administration and use this information to select an appropriate probiotic candidate for each individual (104). Despite these limitations, administration of probiotics is promising for the treatment and prevention of many conditions.

In swine, probiotics are being used for many applications and are an attractive alternative to antibiotics, particularly in newly weaned animals in which the intestinal community has not

fully established itself and is susceptible to disturbance and change (105, 106). Many different species and strains of bacteria are being administered, although the most common strains belong to the genera *Lactobacillus, Bifidobacterium* and *Bacillus* (107). Organisms of these genera are most often used because of their history in association with gut health, their ease of culture, and their regulatory status (most are classified as generally recognized as safe (GRAS) and have low regulatory hurdles). Many studies have shown promising results for the oral administration of probiotic bacteria in swine (108-110); however their effects are not profound and often variable. One study even suggested that administering probiotics at a very high dose can have detrimental effects. *Lactobacillus rhamnosus* administration provided a better outcome in an *Escherichia coli* challenge model, but negative consequences were seen with a high dose (10^{12} cells/day) (111).

Investigation of bacteria for probiotic administration is ongoing, and the more recently developed probiotic strategies have moved away from the administration of a single strain in pure culture. Using probiotic bacteria to ferment feed prior to ingestion by animals has shown great promise, and this strategy gives the probiotic microbes a chance to colonize food particles prior to competition with endogenous intestinal microbes (112). Multi-species probiotic blends are being developed (113), and innovations in the mode of delivery are increasing the viability of probiotics (114). Perhaps the most promising advancement is the realization that probiotic organisms have a greater chance of colonizing the intestinal tract if they are co-administered with the dietary fibers they prefer to consume.

Prebiotics

Any dietary fiber that is consumed in the gut by intestinal bacteria can be considered a prebiotic. If a dietary fiber is not digested by the host but is also not consumed by bacteria in the gut it is not considered a prebiotic. Changes in diet have proven the most effective way to

change the intestinal microbiota, and so prebiotics are an extremely promising way of pushing these communities towards those associated with health (115). Many common dietary fibers that have prebiotic abilities include but are not limited to resistant starches, inulin, xylo-oligosaccharides, and fructo-oligosaccharides. These compounds are all complex carbohydrates that escape host digestion. In the case of some of these compounds, such as inulin or xylo-oligosaccharides, the host lacks the digestive enzymes to cleave the bonds between these various sugars. For others, such as resistant starches, the host technically has the capacity to depolymerize and digest the compounds; however, due to the crystal or physical structure many of the bonds are inaccessible and consequently the molecules escape digestion (99, 115, 116).

Prebiotics affect many members of the intestinal microbiota. The intestinal microbiota is a complex tangle of interconnected food webs, and the introduction of a new substrate causes a domino effect, changing populations of many interdependent bacterial species. It has been shown that certain keystone species are vital for the initial degradation of some prebiotics. Keystone species are those that play an integral role in an ecosystem or ecosystem function, and their presence or absence dramatically affects the ability of other species to succeed in that particular ecosystem. In the case of resistant starches, *Ruminococcus bromii* and various *Bifidobacteria* species have been shown to be keystone species for the degradation of these substrates in colonic ecosystems (117, 118). In both of these scenarios, the initial breakdown of the resistant starch polymer is followed by an increased abundance of bacteria that consume the breakdown products of these keystone degraders. Often the species that benefit are butyrate-producers and as such are associated with the health of the host (119). However, as with probiotics, inter-individual variation is also observed in the literature on resistant starch supplementation. One trial suggested that it is the presence of these keystone starch-degrading

organisms which dictate if an individual will respond favorably to resistant starch supplementation (120).

Resistant starches have been increasingly used in swine agriculture to increase intestinal health and prevent disease. A recent study has detailed the changes in the swine gut microbiota following long-term supplementation of resistant starch and showed an increase in species belonging to the genera *Lachnospiraceae, Prevotella* and *Ruminococcus*, all of which are associated with degradation of dietary fiber, production of SCFAs, and gut health (121). Another study administered resistant starches to weaned pigs in varying concentrations and observed an increase in species belonging to the genus *Bifidobacteria*. This study also suggested that the correct dose of resistant starch is critical and too much can have negative consequences (122). Raw potato starch (RPS) is showing increasing promise for mitigating post-weaning disorders in piglets, with a recent study showing benefits of administering relatively low concentrations (0.5% and 1%) on reducing the incidence of diarrhea (123).

Conclusions

The intestinal microbiota of all animals is an integral part of the host and is tightly connected to health. It is critical to understand these microbial ecosystems and their interactions with the host in order to develop strategies to maximize resistance to disease. The work described in this thesis represents progress towards these goals. Tools to study the butyrate-producing community are outlined and used to investigate this community in the distal gut of swine. A detailed description of a new butyrate-producing bacterial species lays the foundations for its potential use as a probiotic. Raw potato starch's effects on the gut microbiome and host mucosa in weaned pigs are investigated and show promising benefits. These three studies significantly contribute to our goal of increasing knowledge of the swine-gut microbiota and

advance our understanding of how to best manipulate these communities to improve host health and resistance to disease.

Preview of Chapter 1: Tools to study the butyrate producing community in the gut

Because of butyrate's importance in maintaining colonic homeostasis and host health, studying and manipulating the bacterial populations responsible for its production is of great interest. In order to manipulate this community, it is important to understand how butyrate is produced in the gut. Butyrate producers do not form a monophyletic group and at least four different biochemical pathways lead to butyrate production. The cecum and proximal colon are considered to be the main sites of butyrate production in hindgut fermenters; in these locations, carbohydrates that escaped host absorption serve as the main metabolic inputs. The most common pathway in these environments is referred to as the acetyl-CoA pathway. This pathway entails the condensation of two molecules of acetyl-CoA into aceto-acetyl-CoA, then its reduction to crotonyl-CoA and finally butyryl-CoA. Other amino acid-based pathways may be more common in the distal reaches of the colon where fermentable carbohydrates have been depleted (124).

Once butyryl-CoA has been generated, two main mechanisms exist for the final conversion to butyrate. The first is through the phosphorylation of butyryl-CoA and transformation to butyrate, along with the generation of ATP, via butyrate kinase (Buk) (125). The second option, which is much more common in the distal gut, is catalyzed by butyryl-CoA: acetate CoA transferase (But). This enzyme takes the CoA group from butyryl-CoA and transfers it to acetate yielding acetyl-CoA **(Figure 3)**. One of the advantages to using this transferase as the terminal gene of the butyrate synthesis pathway is that it re-generates the initial input of the main butyrate production pathway, acetyl-CoA. This transferase is thought to be especially advantageous in the colonic ecosystem due to the normally high levels of acetate, allowing butyrate producers to take up and utilize a waste product discarded by other microbes (126). Studies have suggested that the majority of butyrate production in the gut is the product of But enzyme activity (124, 125, 127, 128). These characteristics make the *but* gene an excellent marker to study the butyrate-producing community in colonic environments.

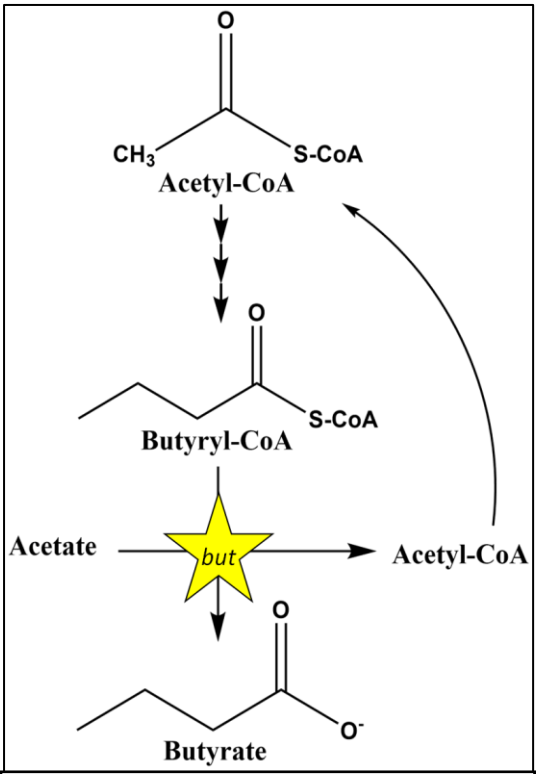

Figure 3: A summarized representation of the most common pathway for butyrate production in gut environments. The gold star depicts But enzyme activity.

Using the *but* gene as a marker for butyrate production is not without challenges. The existence of closely related transferases that have differing substrate preferences (127-129) make it difficult to know which sequences represent legitimate But proteins. Genes encoding 4-hydroxybutyrate transferases (*4hbt*) are the sequences most commonly mistaken for *but* genes;

however, acetyl-CoA hydrolases are often confused as well. Automatic annotation algorithms cannot accurately distinguish between these groups, leading to frequent misannotation of these genes in both genomic and metagenomic datasets (128). Preliminary data suggest that many genomes contain two very similar genes, one that is a verified *but* gene sequence and one that is a closely related transferase but has different substrate specificities and activities. In these cases both genes have very similar conserved regions and are annotated similarly. Unfortunately, because of the existence of these highly conserved regions in related transferases, it is possible that when using degenerate primers non-*but* genes may be amplified along with actual *but* genes. To improve the quality of *but* gene sequence datasets and the automatic annotations of genomes and metagenomes, more research is needed to accurately describe *but* genes and discriminate them from closely related sequences.

This chapter describes the functional characterization of several butyryl-Coa:acetate CoA-transferases from butyrate-producing bacteria isolated from the swine intestinal microbiota. Characterization of these genes' functions and substrate specificities show that functionally-confirmed *but* genes and paralogues exist in the same genome in several species. This functional information is used to identify a discriminating motif between these two groups and this knowledge is used to design a degenerate primer set that preferentially amplifies functional *but* genes over their paralogues. This primer set detects a large number of phylogenetically diverse butyrate producers, but also has significant biases. Finally, this primer set is used to perform high-throughput culture-independent surveys of the butyrate-producing community. This work has been published in the journal Applied and Environmental Microbiology under the title, "Function and Phylogeny of Bacterial Butyryl Coenzyme A:Acetate Transferases and Their Diversity in the Proximal Colon of Swine".

Preview of Chapter 2: Characterization of a butyrate-producing bacterium from the swine intestinal tract and initial evaluations for suitability as a probiotic

Butyrate production is a trait that is found in many different species of bacteria that fill many different ecological niches. Understanding the ecological niches of butyrate-producing bacteria is necessary to manipulate these populations for gut health. Evidence suggests that butyrate-producing bacteria shift their metabolisms, and therefore butyrate production, in response to changing environmental conditions and cohabitating microbes (130). For example, many butyrate producers, such as *Roseburia* sp., expel hydrogen gas as a waste product (131). If the partial pressure of hydrogen gets too high this can cause their metabolism to stall, leading to reduced butyrate production. Hydrogen uptake by methanogenic archaea, such as *Methanobrevibacter smithii,* can lower the partial pressure so that microbial fermentation can occur at optimal efficiency (132). Additionally, some microbes, such as *Megasphaera elsdenii* and *Anaerostipes caccae,* produce butyrate by utilizing lactate from other species and have been shown to increase their butyrate production in co-culture with lactate producers such as *Bifidobacteria* sp. (133-135). Furthermore, evidence indicates that when butyrate producers associate with hydrogen-consuming acetogenic bacteria, the butyrate output of the system increases, such as with the interaction between *Roseburia intestinalis* and *Ruminococcus hydrogenotrophicus* (136). If a bacterial strain is to be used as a probiotic, it is critical to understand it metabolism and potential interactions such as those mentioned above. Only by understanding the ecological context in which butyrate producers exist can strategies be developed to ensure their survival and colonization of the host when administered as a probiotic.

Detailed biochemical and genomic characterizations of bacterial species are the first step in this process. Chapter 2 outlines the description of a new species of bacteria belonging to the genus *Butyricicoccus* and provides a detailed assessment on its required culture conditions and growth characteristics. As part of this analysis a draft genome for this strain has been published and leveraged to improve our understanding of this species and postulate its ecological niche. Finally, this chapter makes some initial assessments of this *Butyricicoccus* strain's (BB10) suitability as a probiotic and potential benefits to the swine host. This work is currently under review in the journal International Journal of Systematic and Evolutionary Microbiology.

Preview of chapter 3: Fueling beneficial host-microbe interactions with raw potato starch

Piglets face a large number of stressors directly after weaning. Weaned piglets experience an abrupt diet change, and their microbial communities must rapidly adapt. During this transition, many of the benefits provided by a healthy microbial community are in flux, and this disturbed microbiota in turn reduces colonization resistance. Piglets with a microbiota better adapted to the new diet experience better health and performance outcomes (increased weight gain, reduced colonization of opportunistic pathogens) (137). Intestinal distresses such as post-weaning diarrhea are common during this transition, and antibiotics are routinely used to combat these disorders (138). However, concerns about the rise of antimicrobial resistance and increased regulation of these compounds necessitate the development of alternative treatments for these issues. Prebiotics such as raw potato starch (RPS) are emerging as promising solutions to intestinal disorders.

RPS is a type 2 resistant starch and has shown great promise as an effective prebiotic. It escapes host digestion by virtue of its large crystalline structure, rendering it physically inaccessible to host enzymes for degrading the glycosidic bonds (139). Once RPS reaches the distal gut it is rapidly fermented by commensal bacteria, and its fermentation has been shown to have a strong butyriogenic effect. RPS is currently being used in some swine production systems, although determining its optimal dosage, mechanism of action, and optimum age administration requires further work.

This chapter outlines the weaning-associated changes in the community composition and functional capabilities of the piglet microbiota. This work shows that dietary RPS can induce beneficial changes in the community composition and functions of the swine gut microbiota as well as its interactions with the host. This work investigates the bacterial food webs responsible for the conversion of RPS to beneficial SCFAs such as butyrate, and suggests that RPS intake is associated with many markers of improved gut health. This study greatly enhances our knowledge of the mechanisms through which RPS is converted to beneficial metabolites and how these metabolites affect host tissues. This work additionally identified microbial species which can be administered with RPS to enhance its effects in the weaned piglet.

Citations

1. Dubilier N, Bergin C, Lott C. 2008. Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. Nature Reviews Microbiology 6:725-740.
2. Douglas AE. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. Annual Reviews Entomology 43:17-37.
3. Lloyd-Price J, Abu-Ali G, Huttenhower C. 2016. The healthy human microbiome. Genome Medicine 8:51.
4. Grice EA, Segre JA. 2011. The skin microbiome. Nature reviews Microbiology 9:244-253.
5. Troccaz M, Gaïa N, Beccucci S, Schrenzel J, Cayeux I, Starkenmann C, Lazarevic V. 2015. Mapping axillary microbiota responsible for body odours using a culture-independent approach. Microbiome 3:3.
6. Smith K, Collier A, Townsend EM, O'Donnell LE, Bal AM, Butcher J, Mackay WG, Ramage G, Williams C. 2016. One step closer to understanding the role of bacteria in diabetic foot ulcers: characterising the microbiome of ulcers. BMC Microbiology 16:54.
7. Huang B, Fettweis JM, Brooks JP, Jefferson KK, Buck GA. 2014. The changing landscape of the vaginal microbiome. Clin Lab Med 34:747-61.
8. Cohen J. 2016. Vaginal microbiome affects HIV risk. Science 353:331.
9. Fernando SC, Purvis HT, Najar FZ, Sukharnikov LO, Krehbiel CR, Nagaraja TG, Roe BA, DeSilva U. 2010. Rumen Microbial Population Dynamics during Adaptation to a High-Grain Diet. Applied and Environmental Microbiology 76:7482-7490.
10. Cone JW, van Gelder AH. 2006. Adaptation of the rumen microbial population to native potato starch degradation determined with the gas production technique and the nylon bag technique. Journal of Animal Physiology and Animal Nutrition (Berl) 90:511-8.
11. Swindle MM, Makin A, Herron AJ, Clubb FJ, Frazier KS. 2011. Swine as Models in Biomedical Research and Toxicology Testing. Veterinary Pathology 49:344-356.
12. Zhang Q, Widmer G, Tzipori S. 2013. A pig model of the human gastrointestinal tract. Gut Microbes 4:193-200.
13. Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A, Laxminarayan R. 2015. Global trends in antimicrobial use in food animals. Proceedings of the National Academy of Sciences 112:5649-5654.
14. Smith TC, Harper AL, Nair R, Wardyn SE, Hanson BM, Ferguson DD, Dressler AE. 2011. Emerging swine zoonoses. Vector Borne Zoonotic Dis 11:1225-34.
15. Peterson LW, Artis D. 2014. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nature Reviews in Immunology 14:141-153.
16. Donaldson GP, Lee SM, Mazmanian SK. 2016. Gut biogeography of the bacterial microbiota. Nature reviews Microbiology 14:20-32.
17. Savage DC. 1977. Microbial ecology of the gastrointestinal tract. Annual Reviews Microbiology 31:107-33.
18. Dukowicz AC, Lacy BE, Levine GM. 2007. Small Intestinal Bacterial Overgrowth: A Comprehensive Review. Gastroenterology & Hepatology 3:112-122.
19. Bures J, Cyrany J, Kohoutova D, Förstl M, Rejchrt S, Kvetina J, Vorisek V, Kopacova M. 2010. Small intestinal bacterial overgrowth syndrome. World Journal of Gastroenterology : WJG 16:2978-2990.
20. Lalezari D. 2012. Gastrointestinal pH profile in subjects with irritable bowel syndrome.

21. Levitt MD. 1970. Oxygen tension in the gut. N Engl J Med 282:1039-40.
22. Zheng L, Kelly CJ, Colgan SP. 2015. Physiologic hypoxia and oxygen homeostasis in the healthy intestine. A Review in the Theme: Cellular Responses to Hypoxia. American Journal of Physiology - Cell Physiology 309:C350-C360.
23. Kelly Caleb J, Colgan Sean P. Breathless in the Gut: Implications of Luminal O_2 for Microbial Pathogenicity. Cell Host & Microbe 19:427-428.
24. Kim YS, Ho SB. 2010. Intestinal Goblet Cells and Mucins in Health and Disease: Recent Insights and Progress. Current Gastroenterology Reports 12:319-330.
25. Spencer J, Klavinskis LS, Fraser LD. 2012. The human intestinal IgA response; burning questions. Front Immunol 3:108.
26. Johansson ME, Larsson JM, Hansson GC. 2011. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proceedings of the National Academy of Sciences USA 108 Suppl 1:4659-65.
27. Johansson MEV, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proceedings of the National Academy of Sciences 105:15064-15069.
28. Marcobal A, Southwick AM, Earle KA, Sonnenburg JL. 2013. A refined palate: bacterial consumption of host glycans in the gut. Glycobiology 23:1038-46.
29. Desai MS, Seekatz AM, Koropatkin NM, Kamada N, Hickey CA, Wolter M, Pudlo NA, Kitamoto S, Terrapon N, Muller A, Young VB, Henrissat B, Wilmes P, Stappenbeck TS, Núñez G, Martens EC. 2016. A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. Cell 167:1339-1353.e21.
30. Pearson JP, Brownlee IA. 2010. The interaction of large bowel microflora with the colonic mucus barrier. International Journal of Inflammation 2010:321426.
31. Donaldson GP, Lee SM, Mazmanian SK. 2016. Gut biogeography of the bacterial microbiota. Nature Reviews in Microbiology 14:20-32.
32. Zilberstein B, Quintanilha AG, Santos MA, Pajecki D, Moura EG, Alves PR, Maluf Filho F, de Souza JA, Gama-Rodrigues J. 2007. Digestive tract microbiota in healthy volunteers. Clinics (Sao Paulo) 62:47-54.
33. Nardone G, Compare D. 2015. The human gastric microbiota: Is it time to rethink the pathogenesis of stomach diseases? United European Gastroenterology Journal 3:255-260.
34. Holman DB, Brunelle BW, Trachsel J, Allen HK. 2017. Meta-analysis To Define a Core Microbiota in the Swine Gut. mSystems 2.
35. Mann E, Schmitz-Esser S, Zebeli Q, Wagner M, Ritzmann M, Metzler-Zebeli BU. 2014. Mucosa-Associated Bacterial Microbiome of the Gastrointestinal Tract of Weaned Pigs and Dynamics Linked to Dietary Calcium-Phosphorus. PLOS ONE 9:e86950.
36. Looft T, Allen HK, Cantarel BL, Levine UY, Bayles DO, Alt DP, Henrissat B, Stanton TB. 2014. Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. The ISME Journal 8:1566-1576.
37. Yang H, Huang X, Fang S, Xin W, Huang L, Chen C. 2016. Uncovering the composition of microbial community structure and metagenomics among three gut locations in pigs with distinct fatness. Scientific Reports 6:27427.
38. Barman NN, Bianchi AT, Zwart RJ, Pabst R, Rothkotter HJ. 1997. Jejunal and ileal Peyer's patches in pigs differ in their postnatal development. Anatomy and Embryology (Berl) 195:41-50.

39. Mowat AM, Agace WW. 2014. Regional specialization within the intestinal immune system. Nat Rev Immunol 14:667-685.
40. Mabbott NA, Donaldson DS, Ohno H, Williams IR, Mahajan A. 2013. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. Mucosal Immunology 6:666-677.
41. Thomas F, Hehemann J-H, Rebuffet E, Czjzek M, Michel G. 2011. Environmental and Gut Bacteroidetes: The Food Connection. Frontiers in Microbiology 2:93.
42. Shankar V, Gouda M, Moncivaiz J, Gordon A, Reo NV, Hussein L, Paliy O. 2017. Differences in Gut Metabolites and Microbial Composition and Functions between Egyptian and U.S. Children Are Consistent with Their Diets. mSystems 2:e00169-16.
43. Nakayama J, Yamamoto A, Palermo-Conde LA, Higashi K, Sonomoto K, Tan J, Lee YK. 2017. Impact of Westernized Diet on Gut Microbiota in Children on Leyte Island. Fronteirs Microbiology 8:197.
44. Girard C, Tromas N, Amyot M, Shapiro BJ. 2017. Gut Microbiome of the Canadian Arctic Inuit. mSphere 2.
45. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature 505:559-563.
46. O'Hara AM, Shanahan F. 2006. The gut flora as a forgotten organ. EMBO Reports 7:688-693.
47. Bull MJ, Plummer NT. 2014. Part 1: The Human Gut Microbiome in Health and Disease. Integrative Medicine: A Clinician's Journal 13:17-22.
48. Van der Waaij D, Berghuis-de Vries JM, Lekkerkerk-van der Wees JEC. 2009. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. Journal of Hygiene 69:405-411.
49. McNeil NI. 1984. The contribution of the large intestine to energy supplies in man. American Journal of Clinical Nutrition 39:338-42.
50. Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. 2012. Microbial degradation of complex carbohydrates in the gut. Gut Microbes 3:289-306.
51. Hehemann J-H, Correc G, Barbeyron T, Helbert W, Czjzek M, Michel G. 2010. Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. Nature 464:908-912.
52. LeBlanc JG, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M. 2013. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Current Opinion in Biotechnology 24:160-168.
53. Magnusdottir S, Ravcheev D, de Crecy-Lagard V, Thiele I. 2015. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. Front Genet 6:148.
54. LeBlanc JG, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M. 2013. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Current Opinion Biotechnology 24:160-8.
55. Said HM. 2013. Recent advances in transport of water-soluble vitamins in organs of the digestive system: a focus on the colon and the pancreas. American Journal of Physiology Gastrointest Liver Physiol 305:G601-10.

56. Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, Tuohy K. 2017. Gut microbiota functions: metabolism of nutrients and other food components. European Journal of Nutrition doi:10.1007/s00394-017-1445-8:1-24.
57. Gustafsson BE, Daft FS, Mc DE, Smith JC, Fitzgerald RJ. 1962. Effects of vitamin K-active compounds and intestinal microorganisms in vitamin K-deficient germfree rats. J Nutr 78:461-8.
58. Frick PG, Riedler G, Brogli H. 1967. Dose response and minimal daily requirement for vitamin K in man. J Appl Physiol 23:387-9.
59. Swann JR, Want EJ, Geier FM, Spagou K, Wilson ID, Sidaway JE, Nicholson JK, Holmes E. 2011. Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. Proceedings of the National Academy of Sciences 108:4523-4530.
60. Ridlon JM, Kang DJ, Hylemon PB, Bajaj JS. 2014. Bile acids and the gut microbiome. Curr Opin Gastroenterol 30:332-8.
61. Ridlon JM, Kang DJ, Hylemon PB. 2006. Bile salt biotransformations by human intestinal bacteria. Journal of Lipid Research 47:241-59.
62. Hoffman JB, Hennig B. 2017. Protective influence of healthful nutrition on mechanisms of environmental pollutant toxicity and disease risks. Annals of the New York Academy of Science doi:10.1111/nyas.13365.
63. Hoffman JB, Hennig B. Protective influence of healthful nutrition on mechanisms of environmental pollutant toxicity and disease risks. Annals of the New York Academy of Sciences doi:10.1111/nyas.13365:n/a-n/a.
64. Mouhid L, Corzo-Martinez M, Torres C, Vazquez L, Reglero G, Fornari T, Ramirez de Molina A. 2017. Improving In Vivo Efficacy of Bioactive Molecules: An Overview of Potentially Antitumor Phytochemicals and Currently Available Lipid-Based Delivery Systems. J Oncol 2017:7351976.
65. Vahid H, Rakhshandeh H, Ghorbani A. 2017. Antidiabetic properties of Capparis spinosa L. and its components. Biomed Pharmacother 92:293-302.
66. Valenzuela-Grijalva NV, Pinelli-Saavedra A, Muhlia-Almazan A, Domínguez-Díaz D, González-Ríos H. 2017. Dietary inclusion effects of phytochemicals as growth promoters in animal production. Journal of Animal Science and Technology 59:8.
67. Daien CI, Pinget GV, Tan JK, Macia L. 2017. Detrimental Impact of Microbiota-Accessible Carbohydrate-Deprived Diet on Gut and Immune Homeostasis: An Overview. Front Immunol 8:548.
68. de Oliveira GLV, Leite AZ, Higuchi BS, Gonzaga MI, Mariano VS. 2017. Intestinal dysbiosis and probiotic applications in autoimmune diseases. Immunology doi:10.1111/imm.12765.
69. Kim D, Zeng MY, Nunez G. 2017. The interplay between host immune cells and gut microbiota in chronic inflammatory diseases. Exp Mol Med 49:e339.
70. Gyuraszova M, Kovalcikova A, Gardlik R. 2017. Association between oxidative status and the composition of intestinal microbiota along the gastrointestinal tract. Med Hypotheses 103:81-85.
71. Rios-Covian D, Ruas-Madiedo P, Margolles A, Gueimonde M, de Los Reyes-Gavilan CG, Salazar N. 2016. Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. Front Microbiol 7:185.
72. Bergman EN. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 70:567-90.

73. Ruppin H, Bar-Meir S, Soergel KH, Wood CM, Schmitt MG, Jr. 1980. Absorption of short-chain fatty acids by the colon. Gastroenterology 78:1500-7.
74. Halestrap AP, Wilson MC. 2012. The monocarboxylate transporter family--role and regulation. IUBMB Life 64:109-19.
75. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud D-J, Bakker BM. 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. Journal of Lipid Research 54:2325-2340.
76. Kim M, Qie Y, Park J, Kim CH. 2016. Gut Microbial Metabolites Fuel Host Antibody Responses. Cell Host Microbe 20:202-14.
77. Donohoe DR, Wali A, Brylawski BP, Bultman SJ. 2012. Microbial regulation of glucose metabolism and cell-cycle progression in mammalian colonocytes. PLoS One 7:e46589.
78. Campbell Y, Fantacone ML, Gombart AF. 2012. Regulation of antimicrobial peptide gene expression by nutrients and by-products of microbial metabolism. European Journal of Nutrition 51:899-907.
79. Hatayama H, Iwashita J, Kuwajima A, Abe T. 2007. The short chain fatty acid, butyrate, stimulates MUC2 mucin production in the human colon cancer cell line, LS174T. Biochem Biophys Res Commun 356:599-603.
80. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. 2008. Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther 27:104-19.
81. Zimmerman MA, Singh N, Martin PM, Thangaraju M, Ganapathy V, Waller JL, Shi H, Robertson KD, Munn DH, Liu K. 2012. Butyrate suppresses colonic inflammation through HDAC1-dependent Fas upregulation and Fas-mediated apoptosis of T cells. Am J Physiol Gastrointest Liver Physiol 302:G1405-15.
82. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 504:451-5.
83. Chang PV, Hao L, Offermanns S, Medzhitov R. 2014. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proc Natl Acad Sci U S A 111:2247-52.
84. Nishio J, Honda K. 2012. Immunoregulation by the gut microbiota. Cell Mol Life Sci 69:3635-50.
85. Duncan SH, Louis P, Thomson JM, Flint HJ. 2009. The role of pH in determining the species composition of the human colonic microbiota. Environ Microbiol 11:2112-22.
86. Cherrington CA, Hinton M, Pearson GR, Chopra I. 1991. Short-chain organic acids at ph 5.0 kill *Escherichia coli* and *Salmonella spp.* without causing membrane perturbation. Journal of Applied Bacteriology 70:161-5.
87. BS R, S V, S S, P D, GP Y, HJ i. 1990. The role of intestinal volatile fatty acids in the *Salmonella* shedding of pigs. Zentralbl Veterinarmed 37:570-4.
88. Rivera-Chavez F, Winter SE, Lopez CA, Xavier MN, Winter MG, Nuccio SP, Russell JM, Laughlin RC, Lawhon SD, Sterzenbach T, Bevins CL, Tsolis RM, Harshey R, Adams LG, Baumler AJ. 2013. *Salmonella* uses energy taxis to benefit from intestinal inflammation. PLoS Pathog 9:e1003267.
89. Nuccio SP, Baumler AJ. 2014. Comparative analysis of *Salmonella* genomes identifies a metabolic network for escalating growth in the inflamed gut. MBio 5:e00929-14.
90. Spees AM, Lopez CA, Kingsbury DD, Winter SE, Baumler AJ. 2013. Colonization resistance: battle of the bugs or me´nage a`trois with the host? PLOS Pathogens 9:1-4.

91. SANDLE GI. 1998. Salt and water absorption in the human colon: a modern appraisal. Gut 43:294-299.
92. Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, Wilson KE, Glover LE, Kominsky DJ, Magnuson A, Weir TL, Ehrentraut SF, Pickel C, Kuhn KA, Lanis JM, Nguyen V, Taylor CT, Colgan SP. 2015. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. Cell Host Microbe 17:662-71.
93. Rivera-Chavez F, Zhang LF, Faber F, Lopez CA, Byndloss MX, Olsan EE, Xu G, Velazquez EM, Lebrilla CB, Winter SE, Baumler AJ. 2016. Depletion of butyrate-producing *Clostridia* from the gut microbiota drives an aerobic luminal expansion of *Salmonella*. Cell Host Microbe 19:443-54.
94. Looft T, Allen HK, Casey TA, Alt DP, Stanton TB. 2014. Carbadox has both temporary and lasting effects on the swine gut microbiota. Front Microbiol 5:276.
95. Taur Y, Xavier JB, Lipuma L, Ubeda C, Goldberg J, Gobourne A, Lee YJ, Dubin KA, Socci ND, Viale A, Perales MA, Jenq RR, van den Brink MR, Pamer EG. 2012. Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. Clin Infect Dis 55:905-14.
96. Haag LM, Fischer A, Otto B, Plickert R, Kuhl AA, Gobel UB, Bereswill S, Heimesaat MM. 2012. Intestinal microbiota shifts towards elevated commensal Escherichia coli loads abrogate colonization resistance against Campylobacter jejuni in mice. PLoS One 7:e35988.
97. Buffie CG, Jarchum I, Equinda M, Lipuma L, Gobourne A, Viale A, Ubeda C, Xavier J, Pamer EG. 2012. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to Clostridium difficile-induced colitis. Infect Immun 80:62-73.
98. Arthur JC, Perez-Chanona E, Muhlbauer M, Tomkovich S, Uronis JM, Fan TJ, Campbell BJ, Abujamel T, Dogan B, Rogers AB, Rhodes JM, Stintzi A, Simpson KW, Hansen JJ, Keku TO, Fodor AA, Jobin C. 2012. Intestinal inflammation targets cancer-inducing activity of the microbiota. Science 338:120-3.
99. Pandey KR, Naik SR, Vakil BV. 2015. Probiotics, prebiotics and synbiotics- a review. Journal of Food Science and Technology 52:7577-7587.
100. Geirnaert A, Wang J, Tinck M, Steyaert A, Van den Abbeele P, Eeckhaut V, Vilchez-Vargas R, Falony G, Laukens D, De Vos M, Van Immerseel F, Raes J, Boon N, Van de Wiele T. 2015. Interindividual differences in response to treatment with butyrate-producing Butyricicoccus pullicaecorum 25-3T studied in an in vitro gut model. FEMS Microbiol Ecol 91.
101. Thomas LV, Ockhuizen T, Suzuki K. 2014. Exploring the influence of the gut microbiota and probiotics on health: a symposium report. Br J Nutr 112 Suppl 1:S1-18.
102. Senan S, Prajapati JB, Joshi CG, Sreeja V, Gohel MK, Trivedi S, Patel RM, Pandya H, Singh US, Phatak A, Patel HA. 2015. Geriatric Respondents and Non-Respondents to Probiotic Intervention Can be Differentiated by Inherent Gut Microbiome Composition. Frontiers in Microbiology 6:944.
103. Martí JM, Martínez-Martínez D, Rubio T, Gracia C, Peña M, Latorre A, Moya A, P. Garay C. 2017. Health and Disease Imprinted in the Time Variability of the Human Microbiome. mSystems 2.

104. Gerritsen J, Smidt H, Rijkers GT, de Vos WM. 2011. Intestinal microbiota in human health and disease: the impact of probiotics. Genes & Nutrition 6:209-240.
105. Hou C, Zeng X, Yang F, Liu H, Qiao S. 2015. Study and use of the probiotic Lactobacillus reuteri in pigs: a review. Journal of Animal Science and Biotechnology 6:14.
106. Yang F, Hou C, Zeng X, Qiao S. 2015. The Use of Lactic Acid Bacteria as a Probiotic in Swine Diets. Pathogens 4:34-45.
107. Kim JC, Hansen CF, Mullan BP, Pluske JR. 2012. Nutrition and pathology of weaner pigs: Nutritional strategies to support barrier function in the gastrointestinal tract. Animal Feed Science and Technology 173:3-16.
108. Modesto M, D'Aimmo MR, Stefanini I, Trevisi P, De Filippi S, Casini L, Mazzoni M, Bosi P, Biavati B. 2009. A novel strategy to select Bifidobacterium strains and prebiotics as natural growth promoters in newly weaned pigs. Livestock Science 122:248-258.
109. Pajarillo EAB, Chae JP, Balolong MP, Kim HB, Park C-S, Kang D-K. 2015. Effects of probiotic Enterococcus faecium NCIMB 11181 administration on swine fecal microbiota diversity and composition using barcoded pyrosequencing. Animal Feed Science and Technology 201:80-88.
110. Yang Y, Zhao X, Le MH, Zijlstra RT, Ganzle MG. 2015. Reutericyclin producing Lactobacillus reuteri modulates development of fecal microbiota in weanling pigs. Front Microbiol 6:762.
111. Li XQ, Zhu YH, Zhang HF, Yue Y, Cai ZX, Lu QP, Zhang L, Weng XG, Zhang FJ, Zhou D, Yang JC, Wang JF. 2012. Risks associated with high-dose Lactobacillus rhamnosus in an Escherichia coli model of piglet diarrhoea: intestinal microbiota and immune imbalances. PLoS One 7:e40666.
112. Missotten JA, Michiels J, Degroote J, De Smet S. 2015. Fermented liquid feed for pigs: an ancient technique for the future. Journal of Animal Science and Biotechnology 6:4.
113. Koyama T, Kirjavainen PV, Fisher C, Anukam K, Summers K, Hekmat S, Reid G. 2010. Development and pilot evaluation of a novel probiotic mixture for the management of seasonal allergic rhinitis. Can J Microbiol 56:730-8.
114. Ross GR, Gusils C, Gonzalez SN. 2008. Microencapsulation of probiotic strains for swine feeding. Biol Pharm Bull 31:2121-5.
115. Louis P, Flint HJ, Michel C. 2016. How to Manipulate the Microbiota: Prebiotics. Adv Exp Med Biol 902:119-42.
116. Gaggia F, Mattarelli P, Biavati B. 2010. Probiotics and prebiotics in animal feeding for safe food production. Int J Food Microbiol 141 Suppl 1:S15-28.
117. Xiaolei Ze FLM, Sylvia H. Duncan, Petra Louis and Harry J. Flint. 2013. Some are more equal than others The role of "keystone" species in the degradation of recalcitrant substrates. Gut Microbes 4:236-240.
118. Scott KP, Martin JC, Duncan SH, Flint HJ. 2014. Prebiotic stimulation of human colonic butyrate-producing bacteria and bifidobacteria, in vitro. FEMS Microbiol Ecol 87:30-40.
119. Rivière A, Selak M, Lantin D, Leroy F, De Vuyst L. 2016. Bifidobacteria and Butyrate-Producing Colon Bacteria: Importance and Strategies for Their Stimulation in the Human Gut. Frontiers in Microbiology 7:979.
120. Venkataraman A, Sieber JR, Schmidt AW, Waldron C, Theis KR, Schmidt TM. 2016. Variable responses of human microbiomes to dietary supplementation with resistant starch. Microbiome 4:33.

121. Umu OC, Frank JA, Fangel JU, Oostindjer M, da Silva CS, Bolhuis EJ, Bosch G, Willats WG, Pope PB, Diep DB. 2015. Resistant starch diet induces change in the swine microbiome and a predominance of beneficial bacterial populations. Microbiome 3:16.
122. Fouhse JM, Ganzle MG, Regmi PR, van Kempen TA, Zijlstra RT. 2015. High Amylose Starch with Low In Vitro Digestibility Stimulates Hindgut Fermentation and Has a Bifidogenic Effect in Weaned Pigs. J Nutr doi:10.3945/jn.115.214353.
123. Heo JM, Agyekum AK, Yin YL, Rideout TC, Nyachoti CM. 2014. Feeding a diet containing resistant potato starch influences gastrointestinal tract traits and growth performance of weaned pigs. Journal Of Animal Science 92:3906-3913.
124. Vital M, Howe AC, Tiedje JM. 2014. Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data. MBio 5:e00889.
125. Louis P, Duncan SH, McCrae SI, Millar J, Jackson MS, Flint HJ. 2004. Restricted distribution of the butyrate kinase pathway among butyrate-producing bacteria from the human colon. Journal of Bacteriology 186:2099-2106.
126. Duncan SH, Barcenilla A, Stewart CS, Pryde SE, Flint HJ. 2002. Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. Applied and Environmental Microbiology 68:5186-5190.
127. Charrier C, Duncan GJ, Reid MD, Rucklidge GJ, Henderson D, Young P, Russell VJ, Aminov RI, Flint HJ, Louis P. 2006. A novel class of CoA-transferase involved in short-chain fatty acid metabolism in butyrate-producing human colonic bacteria. Microbiology 152:179-85.
128. Marius Vital CRP, Qiong Wang, Vincent B Young, Dion A Antonopoulos, Mitchell L Sogin,, Hilary G Morrison LR, Eugene B Chang, Gary B Huffnagle, Thomas M Schmidt, James R Cole, Tiedje aJM. 2013. A gene-targeted approach to investigate the intestinal butyrate-producing bacterial community. Microbiome 1.
129. Louis P, Flint HJ. 2007. Development of a semiquantitative degenerate real-time pcr-based assay for estimation of numbers of butyryl-coenzyme A (CoA) CoA transferase genes in complex bacterial samples. Appl Environ Microbiol 73:2009-12.
130. Shoaie S, Karlsson F, Mardinoglu A, Nookaew I, Bordel S, Nielsen J. 2013. Understanding the interactions between bacteria in the human gut through metabolic modeling. Sci Rep 3:2532.
131. Louis P, Flint HJ. 2009. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol Lett 294:1-8.
132. Nakamura N, Lin HC, McSweeney CS, Mackie RI, Gaskins HR. 2010. Mechanisms of microbial hydrogen disposal in the human colon and implications for health and disease. Annu Rev Food Sci Technol 1:363-95.
133. Sato T, Matsumoto K, Okumura T, Yokoi W, Naito E, Yoshida Y, Nomoto K, Ito M, Sawada H. 2008. Isolation of lactate-utilizing butyrate-producing bacteria from human feces and in vivo administration of *Anaerostipes caccae* strain L2 and galacto-oligosaccharides in a rat model. FEMS Microbiol Ecol 66:528-36.
134. Munoz-Tamayo R, Laroche B, Walter E, Dore J, Duncan SH, Flint HJ, Leclerc M. 2011. Kinetic modelling of lactate utilization and butyrate production by key human colonic bacterial species. FEMS Microbiol Ecol 76:615-24.
135. Belenguer A, Duncan SH, Calder AG, Holtrop G, Louis P, Lobley GE, Flint HJ. 2006. Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. Appl Environ Microbiol 72:3593-9.

136. Chassard C, Bernalier-Donadille A. 2006. H2 and acetate transfers during xylan fermentation between a butyrate-producing xylanolytic species and hydrogenotrophic microorganisms from the human gut. FEMS Microbiol Lett 254:116-22.
137. Han GG, Lee JY, Jin GD, Park J, Choi YH, Chae BJ, Kim EB, Choi YJ. 2017. Evaluating the association between body weight and the intestinal microbiota of weaned piglets via 16S rRNA sequencing. Appl Microbiol Biotechnol doi:10.1007/s00253-017-8304-7.
138. Yancey RJ, Jr., Evans RA, Kratzer DD, Paulissen JB, Carmer SG. 1990. Efficacy of ceftiofur hydrochloride for treatment of experimentally induced colibacillosis in neonatal swine. Am J Vet Res 51:349-53.
139. Ma Z, Boye JI. 2016. Research advances on structural characterization of resistant starch and its structure-physiological function relationship: A review. Critical Reviews in Food Science and Nutrition doi:10.1080/10408398.2016.1230537:1-25.

CHAPTER 2: FUNCTION AND PHYLOGENY OF BACTERIAL BUTYRYL-COA:ACETATE TRANSFERASES AND THEIR DIVERSITY IN THE PROXIMAL COLON OF SWINE

Published in Applied and Environmental Microbiology

Authors: Julian Trachsel[1,2], Darrell O. Bayles[3], Torey Looft[1], Uri Y. Levine[1†], and Heather K. Allen[1*]

Author affiliations:

1 Food Safety and Enteric Pathogens Research Unit, National Animal Disease Center, Agricultural Research Service, Ames, IA 50010, USA

2 Interdepartmental Microbiology Graduate Program, Iowa State University, Ames, IA, 50010, USA

3 Infectious Bacterial Diseases Research Unit, National Animal Disease Center, Agricultural Research Service, Ames, IA 50010, USA

†**Current address:** Novozymes Biologicals, Inc., 5400 Corporate Circle, Salem VA 24153, urlv@novozymes.com

Abstract

Studying the host-associated butyrate-producing bacterial community is important because butyrate is essential for colonic homeostasis and gut health. Previous research has identified the gene encoding butyryl-CoA:acetate transferase (2.3.8.3) as a gene of primary importance for butyrate production in intestinal ecosystems; however, this gene family (*but*) remains poorly defined. We developed tools for the analysis of butyrate-producing bacteria based on twelve putative *but* genes identified in the genomes of nine butyrate-producing bacteria obtained from the swine intestinal tract. Functional analyses revealed that eight of these But proteins had strong But enzyme activity. When *but* paralogues were found within a genome, only one gene per genome encoded strong activity, with the exception of one strain in which no gene encoded strong But activity. Degenerate primers were designed to amplify the functional *but* genes and were tested by amplifying environmental *but* sequences from DNA and RNA extracted from swine colonic contents. The results show diverse *but* sequences from swine-associated butyrate-producing bacteria, most of which clustered near functionally confirmed sequences. Here we describe tools and a framework that allow the bacterial butyrate-producing community to be profiled in the context of animal health and disease.

Importance

Butyrate is a compound produced by the microbiota in the intestinal tracts of animals. This compound is of critical importance for intestinal health, however, there is still much to discover about the bacteria that produce it. Here we present an additional way to study the butyrate-producing community of bacteria. This work will enable researchers to more easily

study this very important bacterial function that has implications for host health and resistance to disease.

Introduction

Short-chain fatty acids (SCFAs) play a central role in the maintenance of colonic homeostasis, which is the delicate balance between the host, its immune system, and the gastrointestinal microbial partners (1). Butyrate in particular has potent effects on host tissues. As with other SCFAs, butyrate is consumed by the host as an energy source; however, unlike the other common SCFAs, such as propionate and acetate, butyrate is the preferred energy source for colonocytes (2) and is rapidly absorbed and used by the colonic epithelium. This rapid oxidation of butyrate reduces local oxygen concentrations, causing the epithelia to become hypoxic and thus limiting the growth of facultative aerobic pathogens such as *Salmonella* (3, 4). In addition, butyrate alters host gene expression for promotion of immune tolerance to the colonic microbiota and to improve the barrier function of the colonic epithelium. For example, butyrate has been shown to increase the secretion of antimicrobial peptides and mucus as well as the expression of tight junction proteins, thickening and strengthening the barrier while making it less hospitable to invasive microbes (5-7). Most of butyrate's immunomodulatory activities result in anti-inflammatory effects, including the production of extra-thymic T-regulatory (T-reg) cells (8), the limitation of pro-inflammatory CD4+ T cell activity (9), the stimulation of epithelial cells to produce retinoic acid (10), and the desensitization of colonic epithelial cells to IFN-γ (11). Although maintenance of immune tolerance is complex and requires a balance among many regulatory factors, butyrate is a major signal for the host immune system leading to inhibition of pro-inflammatory responses and to toleration of microbes that are present (12).

Because of butyrate's importance in maintaining colonic homeostasis and host health, characterizing and manipulating the bacterial populations responsible for its production is of great interest. Butyrate-producing bacteria do not form a monophyletic group, and at least four different fermentation pathways lead to butyrate production (13). The most common pathway for butyrate production in colonic environments entails the condensation of two molecules of acetyl-CoA followed by reduction to butyryl-CoA. After butyryl-CoA has been generated, two different enzymes are responsible for the final conversion to butyrate: butyrate kinase (Buk), and butyryl-CoA: acetate CoA transferase (But) (14), with the But protein being the most common in the colonic environment (13). This enzyme takes the CoA group from butyryl-CoA and transfers it to acetate yielding acetyl-CoA, thus re-generating a substrate of the main butyrate production pathway. This transferase is thought to be especially advantageous in the colonic ecosystem due to the high levels of acetate, allowing butyrate producers to take up and use a waste product of other microbes (15). Many studies have suggested that the majority of butyrate production in hindgut fermenters is the product of But enzyme activity, including in swine (13, 14, 16-18). It should be noted that previous work has suggested not all enzymes capable of But activity are homologous. Previous work demonstrated that some bacteria from clostridial cluster XVI isolated from the chicken cecum had But activity despite lacking genes with significant homology to the *but* gene family (19). The authors identified genes similar to other known propionyl-CoA transferases in these genomes and suggested that these genes were responsible for the observed But activity. This work focuses only on genes encoding But enzymes commonly found in the *Ruminococcaceae* and *Lachnospiracea* families (previously known as clostridial clusters IV and XIVa), and is not applicable to But-active enzymes with different evolutionary origins.

The sequence variation for the *but* gene family is poorly defined and currently includes closely related transferases that have differing substrate preferences (16, 17, 20). The FunGene But-protein database is a large repository of But-like protein sequences and is an excellent resource; however, it contains But proteins and similar transferases that have distinct substrate specificities. Furthermore, few But proteins in this database have been functionally confirmed. Here we have analyzed the *but* gene from previously identified butyrate-producing bacteria from swine (18), defined the functional diversity of the *but* sequences, developed degenerate *but* primers for PCR, and investigated the butyrate-producing bacterial community in the swine colonic environment. The results show that the degenerate *but* primers preferentially amplify genes encoding functional But enzymes over their paralogues, and that diverse *but* genes are transcribed in the swine colon.

Methods

Identifying potential but*-encoding sequences*

Previous work identified nine strains of swine-associated intestinal bacteria as butyrate producers as determined by gas chromatography. Additionally, these strains were also found to exhibit But activity although the active genes could not be identified in all cases (18). These strains were subjected to shotgun genomic sequencing to identify the genes encoding their But activity. Genomic DNAs were isolated using a previously described protocol (21). Sequencing was performed using a HiSeq 2500 sequencer (2 x 150 bp, rapid mode; Illumina, San Diego, CA) or a MiSeq (2 x 300 bp) at the Iowa State University Office of Biotechnology (DNA facility, Ames, IA), and a Pacific Biosciences sequencer (P6-C4 chemistry; PacBio, Menlo Park, CA) at the Yale Center for Genome Analysis (New Haven, CT), and Roche FLX-Titanium chemistry (Roche Diagnostics, Branford, CT, USA). Libraries were prepared according to

manufacturer's directions. The resulting data included some combination of PacBio reads, Roche FLX 2.3-kb mate-pair library reads, and Illumina 7.9-kb mate-pair library reads. These were assembled using the MIRA assembler in a de novo hybrid assembly (22). Potential *but* genes were identified in the genomes by performing a BLAST search with the amino acid sequence of the butyryl-CoA:acetate CoA-transferase gene from *Roseburia intestinalis* L1-82 (Genbank accession: EEV00989).

Testing for butyrate transferase activity

Candidate genes were cloned into the pET-TOPO-101 vector (Invitrogen, Carlsbad, CA) and transformed into TOP-10 *E. coli* chemically competent cells following the manufacturer's instructions, (primers used for cloning are listed in supplementary table 1). Plasmid DNAs were isolated using the MinElute miniprep kit (Qiagen, Valencia, California), and positive clones were confirmed to have full-length gene inserts by sequencing on an Applied Biosystems 3730xl DNA Analyzer (DNA facility, Iowa State University, Ames, IA). Cloned DNAs were additionally transformed into BL-21 star competent cells for protein expression, in accordance with the kit protocol. Cultures (100 mL) were grown for 12 hours in LB containing 50 µg/mL carbenicillin. Expression was induced by adding IPTG to a final concentration of 1 mM. After an additional 6 hours of growth, cultures were harvested by centrifugation, washed, and resuspended in 10mL sterile PBS. Cells were lysed by two passages through a French press (AMINCO, Silver Spring, MD). Lysates were centrifuged at 19,000x g for 10 minutes to remove remaining unlysed cells. Protein expression was confirmed and the amount of recombinant protein in each lysate was estimated by running 15 µg total protein (determined by Bradford assay, (23)) in a 15% SDS-PAGE gel, staining with Coomassie-blue, and comparing the 49kD band to all bands in the

sample using a densitometry analysis in the ImageJ software package (24). Activities were normalized to the amount of protein present in the 49 kD band.

Butyryl CoA-transferase (EC 2.8.3.8) activity was tested using the citrate synthase assay as described (16), and activity was measured with acetate and butyryl-CoA as substrates (Sigma). The acetyl-CoA generated by butyrate transferase is condensed with oxaloacetate, liberating CoASH, which reacts with 5,5'-dithio-bis-(2-nitrobenzoate) to form a yellow thiophenolate anion. The reaction rates were measured by monitoring the absorbance at 412 nm at 39°C on a Beckman DU-650 spectrophotometer (Indianapolis, IN). Crude cell lysates were diluted with sterile water as necessary to achieve the linear range for the rate of the reaction. The reaction was repeated in the absence of acetate to confirm the measured rate was not due to CoA-hydrolase activity.

***Designing and validating conserved primers to* but**

All full-length, functionally validated *but*-like genes were aligned using CLC genomics workbench (Aarhus, Denmark), and conserved regions were identified. Degenerate primers (funbut-FWD, 5': `CARYTIGGIATYGGIGGIATSCC`; funbut-REV, 5': `TGTCCGCCIGYICCRSWRAT`) were designed to preferentially amplify those *but* genes with confirmed activity.

Full-length genes were downloaded from the FunGene *but* database on March 21, 2016, including only those sequences with a score of 275 or higher (25), resulting in 1144 full-length sequences after removing redundant entries. The number of mismatches between the funbut primers and each gene in this dataset was calculated with a Python script utilizing the Biopython libraries (26) (Supplementary Table 1). Previously published primer sets from Flint et al. and

Vital et al. were also analyzed for comparison (17, 20). This script yielded a table listing the number of mismatches to each primer set for each gene entry. Fasttree (27) was used to generate a phylogenetic tree from full-length amino acid sequences, and the R packages APE (28) and ggtree (29) were used to generate primer coverage figures. Sequences considered likely to amplify were those with two or fewer total mismatches to the primers.

To investigate potential amplification biases, the funbut primers were used in qPCR assays to determine which genes are preferentially amplified. Full-length gene amplicons were generated for each gene included in this study (see table 2 for primer sequences). The amplicons were evaluated via Nanodrop (30) and diluted in 2 µg/mL sheared salmon sperm DNA to 10^7 copies/µL. The qPCR reactions were conducted with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) containing each of the funbut primers at 500 nM and 1 µL *but* gene amplicon DNA in 20 µl on a Stratagene 3005P thermocycler (San Diego, CA). Cycling conditions were as follows: 95° C for 30 seconds, 53° C for 30 seconds, 72° for 30 seconds, 40 cycles total. Inferences about amplification preference were made by comparing Ct values for each gene. As each reaction contained the exact same primer concentrations and numbers of target molecules, any difference in the Ct values among the different targets is due to amplification preferences.

MiSeq* but *amplicon library prep

Swine proximal colon contents (10 cm distal from the cecum) were immediately placed in RNALater and quickly homogenized to preserve the integrity of nucleic acids. Samples were subsequently frozen at -80°C until extraction (within one month). DNAs and RNAs were extracted using the PowerClean DNA and RNA extraction kits (Mo Bio, Carlsbad, CA) from

proximal colon contents from six pigs fed a standard diet and associated with a different study (Kumar et al., in review). The iScript Select kit (Bio-Rad, Hercules, CA) was used to generate cDNA from the RNA using random hexamer primers. Amplicon sequencing libraries were prepared following Illumina's 16S Metagenomic Sequencing Library Preparation (Part # 15044223 Rev. B) substituting the funbut primers for the 16S primers. This protocol uses a 2-step PCR procedure: the first step generates the amplicons from environmental samples, and the second step adds the indices and the sequencing adapters. In the first step, the funbut primers were used to amplify a 359 bp fragment using the AccuPrime Taq High Fidelity PCR system (Invitrogen). Due to the inclusion of multiple inosine bases, we were unable to produce PCR product using a proofreading polymerase alone (31, 32) necessitating the use of a procedure that included Taq as well. The first-step PCR reactions contained each primer at 500 nM, 100 ng of template and used an annealing temperature of 53°C for 35 cycles. The second PCR step was performed in accordance with the protocol using KAPA hifi polymerase (KAPA Biosystems, Wilmington, MA) and the Nextera XT v2 indices (Illumina). This library was sequenced on a MiSeq using a 2x300 V3 reagent kit to generate 300 bp paired-end reads.

Sequence analysis

Sequences were processed using mothur (33) following a modified version of the 'Miseq SOP' (http://www.mothur.org/wiki/MiSeq_SOP). Paired-end reads were joined, quality-screened, and aligned to full-length high quality *but* genes downloaded from Fungene as previously mentioned. Sequences passing quality filters were clustered at a 97% similarity cutoff and representative sequences were obtained for each operational taxonomic unit (OTU). These representatives were used in a BLASTn search against a database comprised of full-length sequences from the FunGene *but* gene dataset plus genes from the current work, to determine the

closest matching published sequence (% identity). Communities were subsampled to 1,556 sequences per sample prior to further analysis. Sequences used as references in the phylogenetic trees have had their activities confirmed either in this work or in previously published work. Genes from human strains have been cloned and their activities confirmed in work such as (16, 34). Other work has tested crude cell lysates for But enzyme activity such as (15), and inferred *but* gene presence by measuring butyrate production, acetate consumption and the presence of a gene homologous to confirmed *but* gene sequences. Additionally, reviews such as (35), identify isolates with confirmed But enzyme activity. These reference sequences were trimmed to the length of the representative sequences for the OTUs and used to generate a maximum likelihood tree using RAxML (36).

Data deposition

Bacterial genomes, butyrate transferase sequences and amplicon sequencing data were deposited in Genbank under Bioproject PRJNA341691. The code and data used to generate the figures in this paper are available at https://github.com/Jtrachsel/AEM-funbuts.

Results

Activity encoded by the but *gene is associated with an amino acid sequence motif*

Putative But-encoding genes were identified in the genomic sequence data from the butyrate-producing bacteria isolated from swine. All of the nine genomes analyzed yielded at least one potential *but* gene, and three genomes were predicted to contain two (*Megasphaera*, *Butyricicoccus,* and *Eubacterium)*, resulting in a total of 12 putative But-encoding genes.

Functional analyses revealed that eight genes encoded strong But activity ranging from 7,004 µM/mg*min (strain 27-5-10) to 27,819 µM/mg*min (strain 831b; Table 1). Only one *but* gene per genome showed appreciable activity, with the exception of the *Eubacterium* (strain 68-5-10) in which neither putative But protein was highly active (Table 1). This lack of appreciable But activity in strain 68-5-10 is consistent with previous work with this strain using native whole cell lysate in the same assay (18). All genes that showed strong activity also exhibited similar activity when propionyl-CoA was used as a substrate (Table 1). These results are in agreement with previous characterizations of this gene family (16) and demonstrate that these sequences encode But activity.

Table 1: But enzyme kinetics and amino acid analyses. Sequences with strong activity are shown in bold with grey shading.

Strain of origin	Closest related species (16S BLAST identity)	Gene name	Length (AA)	crude activity (µM/min*mg)		Similarity to Roseburia query sequence (AA)	
				Butyryl-CoA	Propionyl-CoA	%id	%pos
68-3-10	*Eubacterium nodatum (93%)*	68-3-10 #1	*460*	77.3	406.4	47	66
68-3-10	*Eubacterium nodatum (93%)*	68-3-10 #2	*452*	218.1	335.4	48	69
1161	*Megasphaera elsdenii LC-1 (99%)*	*Megasphaera* 1	*441*	655.5	637.2	55	69
1161	***Megasphaera elsdenii LC-1 (99%)***	***Megasphaera* 2**	**448**	**23515.6**	**20747.3**	**49**	**69**
BB10	*Butyricicoccus pullaceacorum (92%)*	*Butyricicoccus* 1	*445*	231.2	785.4	48	67
BB10	***Butyricicoccus pullaceacorum (92%)***	***Butiricicoccus* 2**	**447**	**13367.9**	**12665.3**	**73**	**85**
35-6-1	***Peptoniphilus grossensis (97%)***	**36-5-1**	**447**	**18343.5**	**13495.2**	**62**	**76**
27-5-10	***Intestimonas butyriciprudecens (99%)***	**27-5-10**	**447**	**7004.6**	**5725.3**	**72**	**85**
494a	***Anaerostipes butyraticus (96%)***	**494a**	**446**	**13692.3**	**12767.3**	**74**	**85**
992a	***Anaerostipes hadrus (95%)***	**992a**	**446**	**16152.8**	**12193.9**	**77**	**85**
499	***Roseburia hominis (96%)***	**499**	**446**	**8276.8**	**7946.9**	**83**	**91**
831b	***Roseburia hominis (97%)***	**831b**	**446**	**27819.8**	**27892.9**	**86**	**91**

To determine sequence motifs associated with active But proteins, an amino acid alignment of all 12 putative But proteins was generated. The alignment yielded several differences that demarked those with activity from those without. These differences occurred in a conserved region containing the amino acid motif LQLGIGG (Figure 1). This motif was identical in all highly active But proteins; however, the proteins with low But activity contained at least one substitution in this motif. These data suggested that primers annealing to the nucleic acids encoding this site could be designed to preferentially amplify genes similar to those with high But activity, thus distinguishing genes with potential But activity from non-functional paralogues. The reverse primer-binding site was nondiscriminating since we designed it to a gene region where But and But-like proteins shared similar amino acid residues. Unfortunately no suitable alternative reverse primer-binding site would preferentially amplify all functional genes and still allow a primer of reasonable degeneracy for amplification.

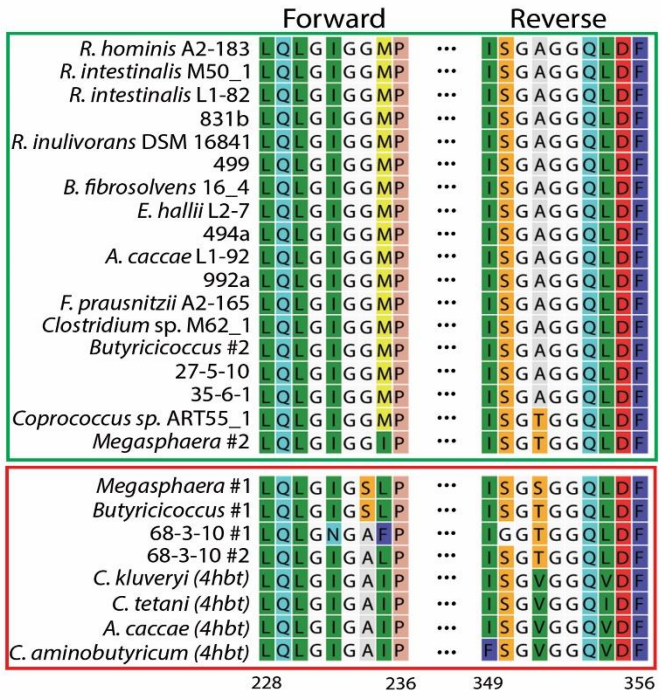

Figure 1: An amino acid alignment of the primer-binding regions. Residue numbering is based on the full length But protein sequence from *Roseburia intestinalis* L1-82 Genbank accession EEV00989. Functionally confirmed sequences occupy the top green-bordered box. Sequences with little activity are bordered by the red box. The glycine at position 234 of this alignment is conserved in all highly active sequences.

Degenerate primers preferentially target function-associated But protein-coding sequences and amplify diverse swine-associated* but *genes

An in-silico analysis compared the primer coverage of currently available *but*-targeting primer sets to the funbut primer pair and revealed that the funbut primers preferentially cover the clade containing all functionally confirmed sequences while having little coverage outside of this clade (Figure 2). The funbut primers are likely to amplify (two or fewer mismatches) 194 sequences, with 95% of these (184 sequences) in the main functional clade of interest. In contrast, the primers published by Vital et al. (17) are likely to amplify 517 sequences, 228 of these being in the clade of interest (44%). Finally, the primers published by Flint et. al. (20) are likely to amplify 5 sequences, all of which are in the main functional clade. It should be noted that the estimates for the number of sequences likely to be amplified are based only on the number of mismatches; amplification conditions also play a large role. Each of these primer sets could amplify more or less diversity than our estimate suggests depending on the exact

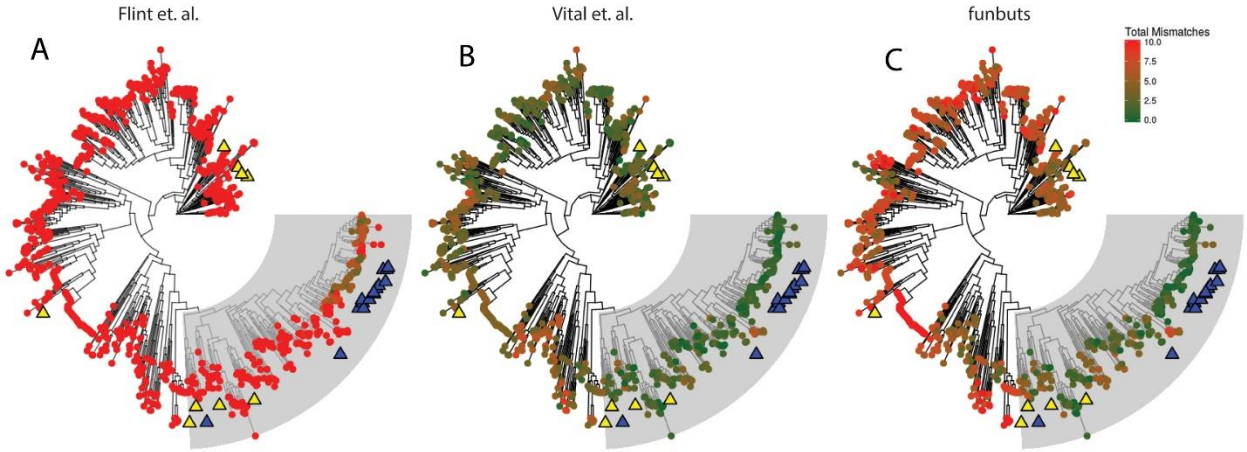

Figure 2: Primer coverage for the three available *but*-targeting primer sets. A: Flint et al. (2), B: Vital et al. (3), C: funbut primers. Maximum-likelihood trees of full-length protein coding sequences from the Fungene *but* database, with the tips of each branch colored to reflect the total number of mismatches each primer set has to that particular sequence (red to green). The clade containing all verified *but* genes is shaded grey. Sequences with confirmed activity are marked with a blue triangle, and *but* paralogues are marked with yellow triangles.

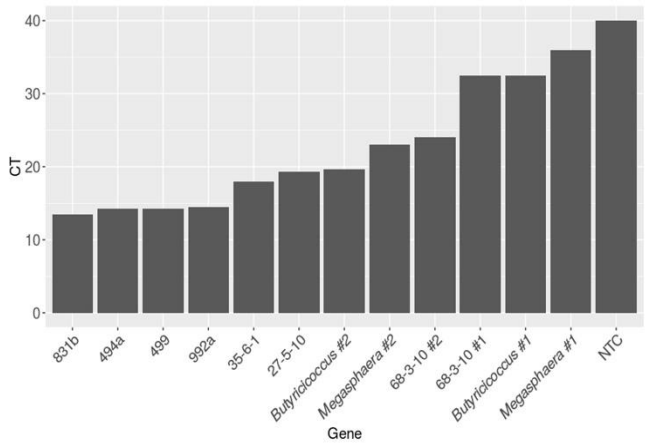

Figure 3: qPCR Ct values using the funbut primers against each gene identified in this study at 10^7 target molecules per reaction. Genes with lower Ct values are preferentially amplified over those with higher Ct values. NTC, no template control

conditions of the PCR reaction. These results show that the funbut primers are more specific to diverse genes encoding functional Buts than are previously published *but* primers.

The in silico validation of the funbut primers suggests that they will preferentially amplify functional *but* genes over their paralogues, and to verify these findings we investigated the amplification preference of our primers for the functionally validated But-encoding genes in this study. The funbut primers preferentially amplified sequences associated with But enzyme function over sequences associated with little or no But enzyme activity (Figure 3). However, some *but* genes were amplified in fewer qPCR cycles and therefore more readily than others, revealing amplification biases even among the functionally confirmed genes (Figure 3).

A large, unexplored diversity of but *genes exists in the swine hindgut*

The funbut primers were applied to nucleic acids from a gut microbial community to evaluate *but* gene detection in this ecosystem. Diverse *but* genes were amplified from total DNA

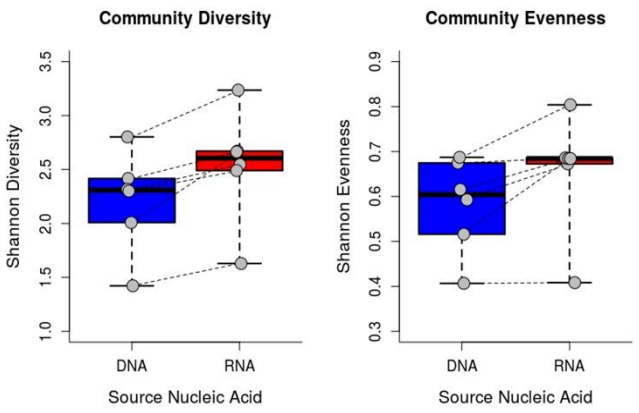

Figure 4: Shannon diversity and evenness indices of DNA and RNA *but* gene sequences, with communities from the same animal joined by a dotted line.

and RNA from swine proximal colonic contents. The colonic contents of six pigs yielded 90 OTUs from total DNA and 86 OTUs from total RNA (92 total unique OTUs, 97% similarity). Several OTUs were present in all animals, but these OTUs differed depending on which nucleic acid was used to profile the community. 14 OTUs were detected in RNA libraries from every pig, four OTUs were detected in all DNA libraries, and three OTUs were detected in every library regardless of nucleic acid type (OTU4, OTU14, and OTU23). The RNA-based communities harbored a greater diversity of *but* sequences, and these communities were more even when compared with the DNA-based communities from the same sample (Figure 4; Shannon diversity index: Wilcoxon paired, p=0.03; Shannon evenness: Wilcoxon paired, p=0.03). Similarly, community membership tended to differ between the RNA and DNA-based communities (Figure 5), suggesting that the most active butyrate producers may not be the most abundant.

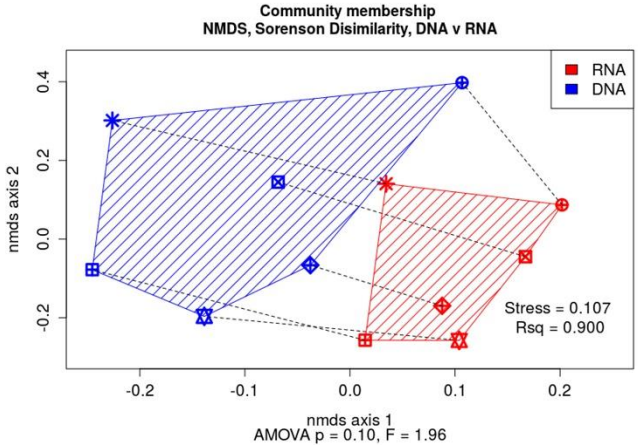

Figure 5: Non-metric multidimensional scaling plot of Sorenson dissimilarity distances (membership) of the *but* gene sequence communities from six swine colons. Communities from the same animal are joined with a dotted line.

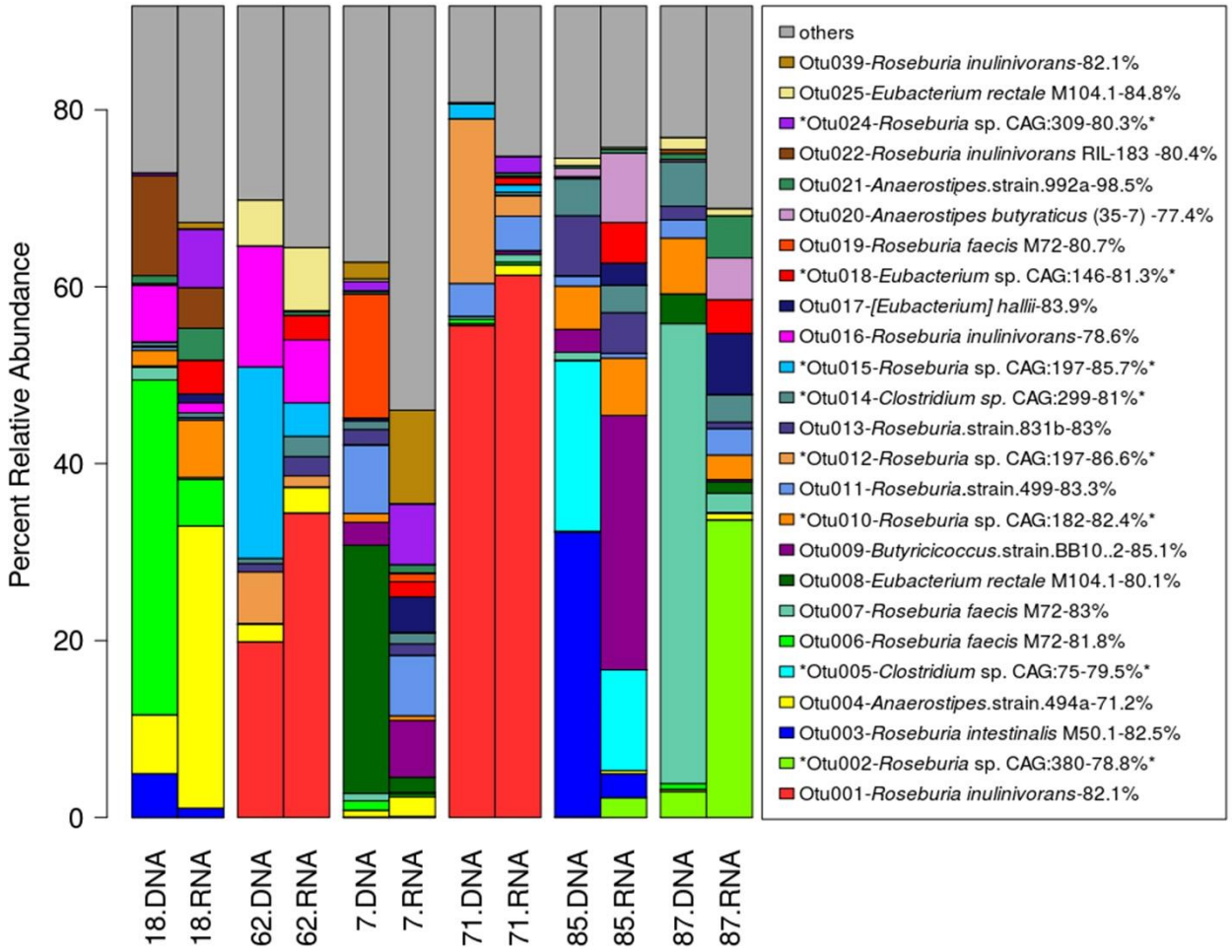

Figure 6: Twenty-five most abundant OTUs, clustered at 97% similarity at the DNA level. OTU designations are followed by the names of the genomes containing the top homologues of representative sequences for each cluster, followed by percent identity (top BLAST hit). Asterisks denote genomes assembled from fecal metagenomes. Target sequences for this BLAST search were from the FunGene *but* gene database as well as the genes identified in this work.

Representative sequences of many of the OTUs differed greatly from previously known, cultured butyrate producers, and some showed more similarity to *but* genes from organisms only detected in metagenomes (Figure 6). In total, 33 out of 92 OTUs were represented by sequences that most closely matched organisms only detected in metagenomic datasets. Similarly, many OTUs were represented by sequences with relatively low identity to any previously detected *but* gene. The maximum identity detected was 100% and the minimum

was 71.2% (OTU4). Representative sequences from 82 OTUs showed < 90% identity, and 23 showed <80% identity to previously detected genes in the reference databases, whether they were of metagenomic origin or not. These findings suggest that many as-yet-uncultured butyrate-producers exist in the swine gut and that this community is underrepresented in databases. Furthermore, the abundances of many OTUs from the same animal differed greatly depending on whether gene copy abundance (DNA) or transcript copy abundance (RNA) was considered (Figure 6), supporting the idea that the transcriptionally active population is distinct from the most abundant.

Predicting function from phylogenetic analysis of But protein sequences

The genes encoding highly functional But proteins are phylogenetically separated from potential paralogues, but this separation is not perfect (Figure 7), with some verified But enzymes and potential paralogues occupying the same clades on the tree. An example of this is the *Megasphaera* #2 gene, which encoded stronger But enzyme activity than the paralogue Megasphaera #1 gene from the same genome but was more divergent from the large functional clade than other sequences with confirmed activity. Phylogenetic placement was used to determine whether the OTUs detected by the funbut primer set encoded highly functional But enzymes or paralogues with lower activity. The representative DNA sequence from each OTU was aligned with confirmed *but* DNA sequences from the literature and this work (trimmed to the amplicon length), and a maximum likelihood phylogenetic tree was constructed from this alignment (Figure 8). Similar to the tree constructed with full-length sequences, most of the confirmed reference sequences grouped together in one main clade apart from potential *but*

paralogues, with the Megasphaera #2 gene being the exception. The vast majority of the OTUs detected by the funbut primers clustered more closely with *but* sequences encoding highly-active enzymes than with sequences encoding low activity. Out of 18,672 total sequences, 18,559 (99.4%) were contained within OTUs in the main functional clade while only 113 (0.6%) were outside this clade near potential *but* paralogues. The results suggest that phylogenetic relatedness is predictive of function for the majority of *but* gene sequences and that the funbut primers preferentially amplify functional *but* genes. However, function is more difficult to predict for distantly related, deeply branching sequences within the *but* gene family.

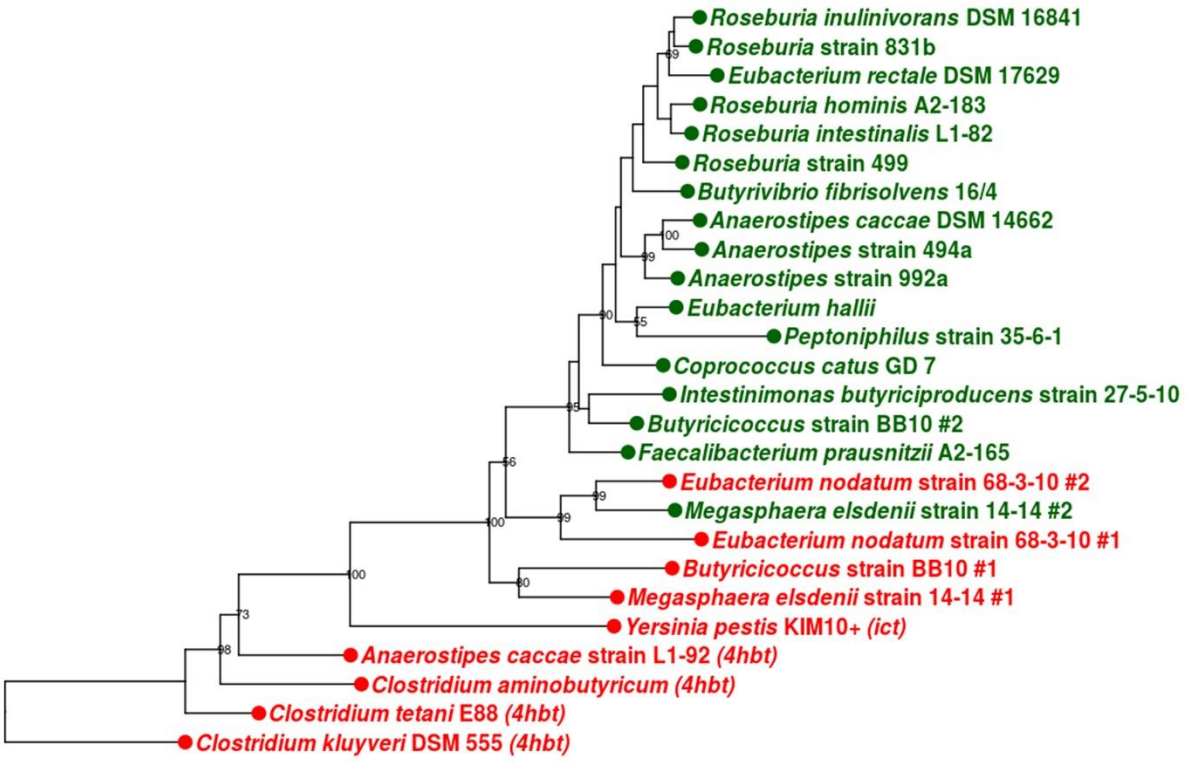

Figure 7: Maximum likelihood tree containing full-length protein-coding sequences. Sequences with confirmed function are shown in green, and potential paralogues are shown in red. Note how functional But-encoding sequences are interspersed with paralogues towards the root of the tree.

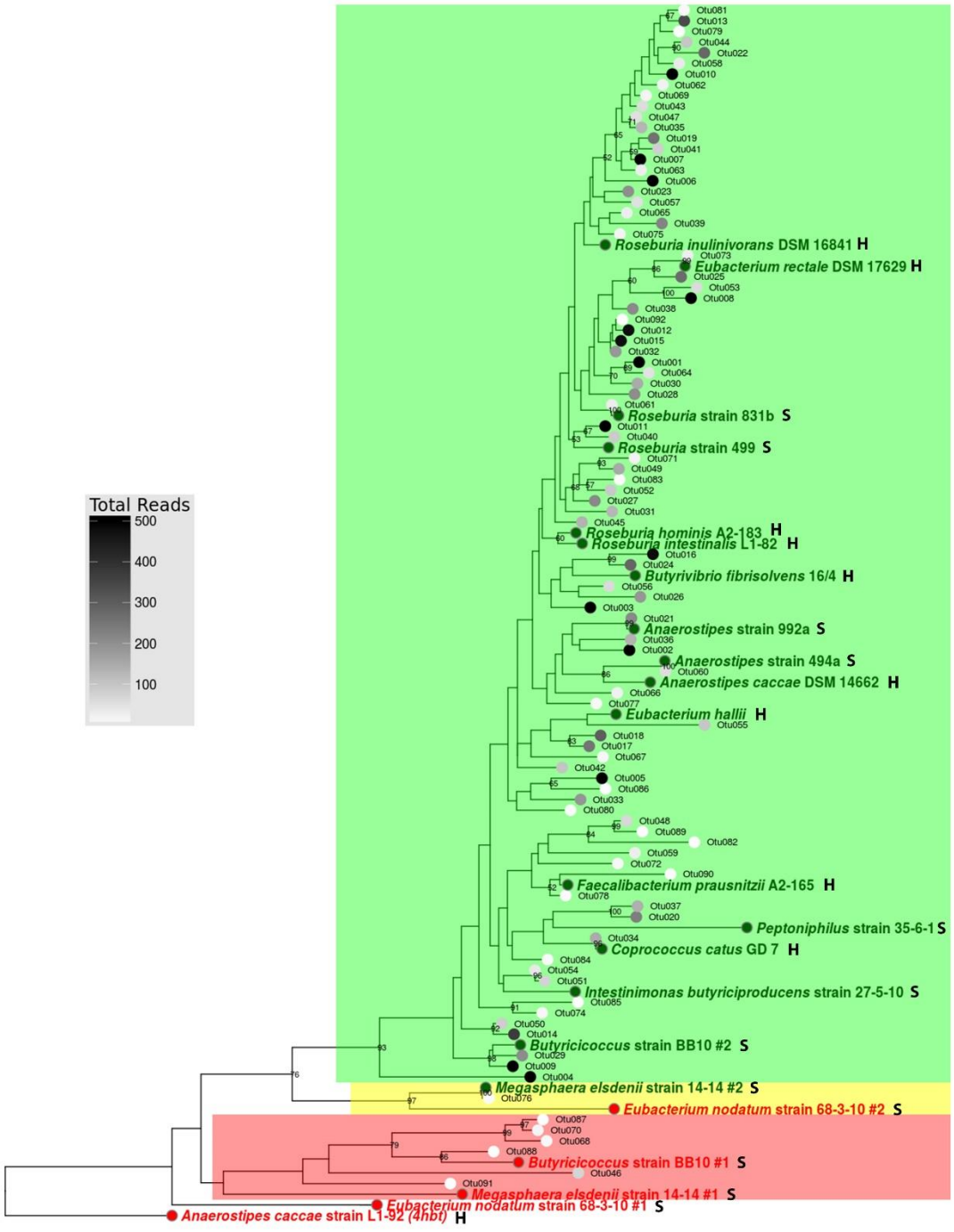

Figure 8: A phylogenetic tree (maximum likelihood) of representative sequences from OTUs from this study, and previously studied But-like sequences identified in Vital et al., 2014 (1). OTUs were generated at 97% similarity with the funbut primers. Sequences with confirmed But enzyme activity are shown in green, and sequences that have failed to demonstrate activity in functional assays are shown in red. Numbers at the nodes are bootstrap values after 1000 resamplings. The main clade containing all functionally confirmed reference sequences is shaded green, the clade containing both confirmed But-encoding genes and potential paralogues is shaded yellow, the clade containing only potential paralogues with low activity is shaded red. Reference sequences from humans are marked with an H, and those from swine with an S.

Discussion

Butyrate is centrally important to colonic homeostasis and is present in every vertebrate gut system studied to date (37). The butyrate-producing microbiota has been implicated in host health in many different disease models (38-48), resulting in increased interest in studying this community. It is therefore important to have a reliable tool to identify butyrate-producing microbes and to detect their activity. The *but* gene is an excellent candidate for this probe due to its ubiquity in colonic environments, and we show the rational design of a primer set that detects *but* genes associated with butyrate transferase function. However, any analysis of the butyrate-producing community that only examines the *but* gene cannot be considered exhaustive. The bacterial butyrate synthesis pathway can be completed by other proteins such as Buk and Ato, as well as non-homologous enzymes capable of But activity (13, 19, 49). Characterizing a wide variety of functional genes is a critical step necessary to design targeted primers and probes. These data are additionally valuable when conducting comparative analyses of amplicon and metagenomic datasets from gut bacterial communities under different conditions.

Comparison of currently available but *primer sets*

Several primer sets targeting the *but* gene are currently available. The first was described by Flint et al. in 2007 (20). This primer set was designed to be used in qPCR assays to estimate the total number of *but* gene copies in complex environments such as feces. Much care was taken to avoid any amplification of non-target, closely related transferases, resulting in a conservative primer set. These primers are unlikely to amplify paralogues of the *but* gene, but also miss much of the full diversity of functionally verified gene products. Due to the specificity

of these primers, very little spurious amplification is observed when used on complex samples such as feces.

Conversely, Vital et al. recently described a more promiscuous set of *but* primers (17). These primers were designed to amplify a wide range of *but*-like sequences, acknowledging that they would amplify closely related non-*but* genes. The primers were used to elucidate the diversity of *but* gene sequences in humans and many other vertebrate species via Roche's 454 pyrosequencing (17, 37). The in silico processing pipeline they describe attempts to remove some of the non-*but* sequences by eliminating those that closely match reference sequences that reside outside of the phylogenetic cluster formed by functionally confirmed But-encoding genes. This method, while useful, is imperfect; the functional validation of *but* gene family members presented here has revealed that the phylogenetic separation of verified But-encoding genes and their paralogues is not absolute (Figures 7,8). Additionally, because of the degeneracy of this primer set, spurious off-target amplification and incorrectly sized PCR products regularly occurs with these primers, necessitating the inclusion of a gel extraction step in sequencing library prep (17, 37).

The development of the funbut primer set built upon these two approaches by amplifying functionally verified yet diverse *but* gene sequences. The funbut primer set amplifies a higher diversity of *but* genes than the primers described by Flint et al., and fewer non-*but* paralogues than the primers described by Vital et al. However, as is the case with most primer sets, these will likely miss or under represent some important groups. For example, although we detected some *but* sequences similar to those of *Faecalibacterium*, these appeared at a much lower abundance than would be expected from 16S rRNA gene sequence-based studies of the swine gut. Importantly, due to the increased specificity of the funbut primers compared to the Vital et

al. primers, no incorrectly sized PCR product has been observed when amplifying from complex fecal or mucosal samples, even at annealing temperatures as low as 45 C. This allows for the omission of the gel extraction step when preparing sequencing libraries, and for the possibility of using these primers in SYBR-based qPCR assays.

The active members of the butyrate-producing community differ from the abundant members

The new *but* primers detected striking differences in community composition from the same starting material (proximal colon contents) depending on whether DNA or RNA was used as the template. Sequences detected in DNA-derived libraries are not necessarily being transcribed and translated into proteins, and may be representative of microbes simply passing through the intestinal tract, or those utilizing an alternative metabolism not involving butyrate production. At best, DNA-based *but* libraries represent the functional potential in the ecosystem. In contrast, sequences detected in RNA-based libraries represent microbes that are actively transcribing *but* genes. These active microbes represent a subset of the total *but*-containing community; however, we detected a greater diversity of *but* genes in the RNA-based libraries than in the DNA-based libraries. Other studies have identified similar differences between the metagenome and metatranscriptome, such as the observation that functional genes for methanogenesis in the human gut were far more abundant in the metatranscriptome compared to the metagenome (50). This reinforces the idea that the gut ecosystem contains microbes that may be in low abundance but are highly active, and that DNA-based studies may overlook their importance. When profiling the butyrate-producing community, RNA may be a more appropriate source molecule rather than DNA.

The phylogenetic relatedness of But protein sequences informs potential function

Within the currently defined But protein family there are genes encoding functional But enzymes and very similar paralogues. Both of these groups are more similar to each other than to the next most similar gene family, the 4-hydroxybutyrate transferases. One large clade harbored the vast majority of functionally confirmed *but* genes as well as the majority of all *but*-related OTUs detected, supporting the use of phylogenetic analyses to predict function for this family of But proteins. Outside of this clade, functionally confirmed and unconfirmed But enzymes were interspersed (Figures 7,8). However, this work does not rule out the possibility that paralogues also exist in the main functional clade as well. Because But enzyme activity is advantageous in colonic ecosystems, it is possible that enzymes specializing in this function have evolved multiple times from different ancestor proteins. Work by Eeckhaut et al. identified But enzyme activity from bacteria that lacked genes similar to *but* genes or their paralogues (19). They proposed that genes most similar to propionyl-CoA transferases were responsible for But enzyme activity in these organisms. Additional discovery and analysis of butyrate-producing organisms is required to delineate the full functional sequence diversity of deeply branching But protein sequences and identify other protein families capable of But enzyme activity.

Further emphasizing the need to more fully characterize this family, and in agreement with previous gene-targeted studies, (17, 37) we detected many OTUs with low identity to both confirmed sequences and cultured organisms. This work reveals many gaps in our knowledge of the *but* gene family. Due to the importance of this bacterial function in nearly all colonic ecosystems, better characterization of this community is necessary. It follows that culturing novel butyrate-producers and identifying their functional genes remains and important step to improve *but* datasets.

Potential Identities of some* but *gene paralogues

Many similar fatty acid CoA-transferases are easily confused for *but* genes. The 4-hydroxybutyryl-CoA: acetate CoA-transferases (4-*hbt*) are known to be similar; however, several researchers have been investigating genes that are more similar to *but* genes than 4-*hbt* genes and are required for full pathogenicity in *Yersinia pestis* and *Salmonella* (51). These genes have been proposed to be itaconate –CoA transferases. They transfer a CoA group from succinyl-CoA onto itaconate, thus activating it and enabling its degradation into acetyl-CoA and pyruvate. Indeed, many entries in the Fungene database for *but*-like genes are from *Salmonella* and *Yersinia* genomes. These genes cluster more closely with functionally confirmed *but* genes than to the 4-*hbt* genes. It is likely that many other genes that closely resemble *but* genes act to move CoA moieties among various fatty acids.

Analyses of butyryl-CoA transferases in the animal intestinal ecosystem enable the study of a functional aspect of the gut microbiota and how it relates to health and disease. This research provides a tool to investigate functional butyrate transferases in the swine gut microbiota, and could also be applied to other animals, other environmental samples, or could be used to generate *but* amplicon data sets from metagenomic samples. This advances the analyses of the host-associated butyrate-producing community for enhancing swine health and improving food safety.

Acknowledgements

We thank Sam Humphrey, Stephanie Jones, Joel Nott, Thad Stanton, Lisa Lai, Jennifer Jones, and David Alt for technical support and helpful advice. We appreciate the caretakers in the Animal Resources Unit for managing the animals. Julian Trachsel was supported by a

fellowship from the Office of Biotechnology, Iowa State University. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S Department of Agriculture. USDA is an equal opportunity provider and employer.

Citations

1. **Hooper LV, Littman DR, Macpherson AJ.** 2012. Interactions between the microbiota and the immune system. Science **336:**1268-1273.
2. **Donohoe DR, Wali A, Brylawski BP, Bultman SJ.** 2012. Microbial regulation of glucose metabolism and cell-cycle progression in mammalian colonocytes. PLoS One **7:**e46589.
3. **Rivera-Chavez F, Zhang LF, Faber F, Lopez CA, Byndloss MX, Olsan EE, Xu G, Velazquez EM, Lebrilla CB, Winter SE, Baumler AJ.** 2016. Depletion of butyrate-producing *Clostridia* from the gut microbiota drives an aerobic luminal expansion of *Salmonella*. Cell Host Microbe **19:**443-454.
4. **Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, Wilson KE, Glover LE, Kominsky DJ, Magnuson A, Weir TL, Ehrentraut SF, Pickel C, Kuhn KA, Lanis JM, Nguyen V, Taylor CT, Colgan SP.** 2015. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. Cell Host Microbe **17:**662-671.
5. **Campbell Y, Fantacone ML, Gombart AF.** 2012. Regulation of antimicrobial peptide gene expression by nutrients and by-products of microbial metabolism. Eur J Nutr **51:**899-907.
6. **Hatayama H, Iwashita J, Kuwajima A, Abe T.** 2007. The short chain fatty acid, butyrate, stimulates MUC2 mucin production in the human colon cancer cell line, LS174T. Biochem Biophys Res Commun **356:**599-603.
7. **Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ.** 2008. Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther **27:**104-119.
8. **Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY.** 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature **504:**451-455.
9. **Fontenelle B, Gilbert KM.** 2012. n-Butyrate anergized effector CD4+ T cells independent of regulatory T cell generation or activity. Scand J Immunol **76:**457-463.
10. **Schilderink R, Verseijden C, Seppen J, Muncan V, van den Brink GR, Lambers TT, van Tol EA, de Jonge WJ.** 2016. The SCFA butyrate stimulates the epithelial production of retinoic acid via inhibition of epithelial HDAC. Am J Physiol Gastrointest Liver Physiol **310:**G1138-1146.
11. **Zimmerman MA, Singh N, Martin PM, Thangaraju M, Ganapathy V, Waller JL, Shi H, Robertson KD, Munn DH, Liu K.** 2012. Butyrate suppresses colonic inflammation through HDAC1-dependent Fas upregulation and Fas-mediated apoptosis of T cells. Am J Physiol Gastrointest Liver Physiol **302:**G1405-1415.
12. **Nishio J, Honda K.** 2012. Immunoregulation by the gut microbiota. Cell Mol Life Sci **69:**3635-3650.
13. **Vital M, Howe AC, Tiedje JM.** 2014. Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data. MBio **5:**e00889.
14. **Louis P, Duncan SH, McCrae SI, Millar J, Jackson MS, Flint HJ.** 2004. Restricted distribution of the butyrate kinase pathway among butyrate-producing bacteria from the human colon. Journal of Bacteriology **186:**2099-2106.

15. **Duncan SH, Barcenilla A, Stewart CS, Pryde SE, Flint HJ.** 2002. Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. Applied and Environmental Microbiology **68:**5186-5190.
16. **Charrier C, Duncan GJ, Reid MD, Rucklidge GJ, Henderson D, Young P, Russell VJ, Aminov RI, Flint HJ, Louis P.** 2006. A novel class of CoA-transferase involved in short-chain fatty acid metabolism in butyrate-producing human colonic bacteria. Microbiology **152:**179-185.
17. **Marius Vital CRP, Qiong Wang, Vincent B Young, Dion A Antonopoulos, Mitchell L Sogin,, Hilary G Morrison LR, Eugene B Chang, Gary B Huffnagle, Thomas M Schmidt, James R Cole, Tiedje aJM.** 2013. A gene-targeted approach to investigate the intestinal butyrate-producing bacterial community. Microbiome **1**.
18. **Levine UY, Looft T, Allen HK, Stanton TB.** 2013. Butyrate-producing bacteria, including mucin degraders, from the swine intestinal tract. Appl Environ Microbiol **79:**3879-3881.
19. **Eeckhaut V, Van Immerseel F, Croubels S, De Baere S, Haesebrouck F, Ducatelle R, Louis P, Vandamme P.** 2011. Butyrate production in phylogenetically diverse Firmicutes isolated from the chicken caecum. Microb Biotechnol **4:**503-512.
20. **Louis P, Flint HJ.** 2007. Development of a semiquantitative degenerate real-time pcr-based assay for estimation of numbers of butyryl-coenzyme A (CoA) CoA transferase genes in complex bacterial samples. Appl Environ Microbiol **73:**2009-2012.
21. **Wilson K.** 1987. Preparation of genomic DNA from bacteria. Current protocols in molecular biology**:**2.4. 1-2.4. 5.
22. **Chevreux B, Wetter T, Suhai S.** Genome sequence assembly using trace signals and additional sequence information, p 45-56. *In* (ed),
23. **Bradford MM.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72:**248-254.
24. **Schneider CA, Rasband WS, Eliceiri KW.** 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods **9:**671-675.
25. **Fish JA, Chai B, Wang Q, Sun Y, Brown CT, Tiedje JM, Cole JR.** 2013. FunGene: the functional gene pipeline and repository. Front Microbiol **4:**291.
26. **Cock PJ, Antao T, Chang JT, Chapman BA, Cox CJ, Dalke A, Friedberg I, Hamelryck T, Kauff F, Wilczynski B, de Hoon MJ.** 2009. Biopython: freely available Python tools for computational molecular biology and bioinformatics. Bioinformatics **25:**1422-1423.
27. **Price MN, Dehal PS, Arkin AP.** 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol **26:**1641-1650.
28. **Paradis E, Claude J, Strimmer K.** 2004. APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics **20:**289-290.
29. **Yu G, Smith D, Zhu H, Guan Y, Lam T.** 2016. ggtree: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods in Ecology and Evolution **2041**.
30. **Desjardins P, Conklin D.** 2010. NanoDrop Microvolume Quantitation of Nucleic Acids. Journal of Visualized Experiments : JoVE doi:10.3791/2565**:**2565.

31. **Fujiwara H, Fujiwara K, Hashimoto K.** 1995. PCR with deoxyinosine-containing primers using DNA polymerases with proofreading activity. PCR Methods Appl **4:**239-240.
32. **Knittel T, Picard D.** 1993. PCR with degenerate primers containing deoxyinosine fails with Pfu DNA polymerase. PCR Methods Appl **2:**346-347.
33. **Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD.** 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol **79:**5112-5120.
34. **Louis P, McCrae SI, Charrier C, Flint HJ.** 2007. Organization of butyrate synthetic genes in human colonic bacteria: phylogenetic conservation and horizontal gene transfer. FEMS Microbiol Lett **269:**240-247.
35. **Louis P, Hold GL, Flint HJ.** 2014. The gut microbiota, bacterial metabolites and colorectal cancer. Nat Rev Micro **12:**661-672.
36. **Stamatakis A.** 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics **30:**1312-1313.
37. **Vital M, Gao J, Rizzo M, Harrison T, Tiedje JM.** 2015. Diet is a major factor governing the fecal butyrate-producing community structure across Mammalia, Aves and Reptilia. ISME J **9:**832-843.
38. **Cerisuelo A, Marin C, Sanchez-Vizcaino F, Gomez EA, de la Fuente JM, Duran R, Fernandez C.** 2014. The impact of a specific blend of essential oil components and sodium butyrate in feed on growth performance and Salmonella counts in experimentally challenged broilers. Poult Sci **93:**599-606.
39. **Metzler-Zebeli BU, Hooda S, Pieper R, Zijlstra RT, van Kessel AG, Mosenthin R, Ganzle MG.** 2010. Nonstarch polysaccharides modulate bacterial microbiota, pathways for butyrate production, and abundance of pathogenic Escherichia coli in the pig gastrointestinal tract. Appl Environ Microbiol **76:**3692-3701.
40. **Khan S, Jena GB.** 2014. Protective role of sodium butyrate, a HDAC inhibitor on beta-cell proliferation, function and glucose homeostasis through modulation of p38/ERK MAPK and apoptotic pathways: Study in juvenile diabetic rat. Chem Biol Interact **213:**1-12.
41. **Kumar A, Alrefai WA, Borthakur A, Dudeja PK.** 2015. Lactobacillus acidophilus counteracts enteropathogenic E. coli-induced inhibition of butyrate uptake in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol doi:10.1152/ajpgi.00186.2015**:**ajpgi 00186 02015.
42. **Kolho K-L, Korpela K, Jaakkola T, Pichai MVA, Zoetendal EG, Salonen A, de Vos WM.** 2015. Fecal Microbiota in Pediatric Inflammatory Bowel Disease and Its Relation to Inflammation. Am J Gastroenterol **110:**921-930.
43. **Chiba M, Tsuji T, Nakane K, Komatsu M.** 2015. High amount of dietary fiber not harmful but favorable for Crohn disease. Perm J **19:**58-61.
44. **Louis P, Hold GL, Flint HJ.** 2014. The gut microbiota, bacterial metabolites and colorectal cancer. Nat Rev Microbiol **12:**661-672.
45. **Cushing K, Alvarado DM, Ciorba MA.** 2015. Butyrate and Mucosal Inflammation: New Scientific Evidence Supports Clinical Observation. Clin Transl Gastroenterol **6:**e108.

46. **Chassard C, Dapoigny M, Scott KP, Crouzet L, Del'homme C, Marquet P, Martin JC, Pickering G, Ardid D, Eschalier A, Dubray C, Flint HJ, Bernalier-Donadille A.** 2012. Functional dysbiosis within the gut microbiota of patients with constipated-irritable bowel syndrome. Aliment Pharmacol Ther **35:**828-838.
47. **Russell WR, Gratz SW, Duncan SH, Holtrop G, Ince J, Scobbie L, Duncan G, Johnstone AM, Lobley GE, Wallace RJ, Duthie GG, Flint HJ.** 2011. High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. Am J Clin Nutr **93:**1062-1072.
48. **Stoll ML, Kumar R, Morrow CD, Lefkowitz E, Cui X, Genin A, Cron RQ, Elson CO.** 2014. Altered microbiota associated with abnormal humoral immune responses to commensal organisms in enthesitis-related arthritis. Arthritis Research & Therapy **16**.
49. **Bui TP, Ritari J, Boeren S, de Waard P, Plugge CM, de Vos WM.** 2015. Production of butyrate from lysine and the Amadori product fructoselysine by a human gut commensal. Nat Commun **6:**10062.
50. **Franzosa EA, Morgan XC, Segata N, Waldron L, Reyes J, Earl AM, Giannoukos G, Boylan MR, Ciulla D, Gevers D, Izard J, Garrett WS, Chan AT, Huttenhower C.** 2014. Relating the metatranscriptome and metagenome of the human gut. Proceedings of the National Academy of Sciences **111:**E2329-E2338.
51. **Sasikaran J, Ziemski M, Zadora PK, Fleig A, Berg IA.** 2014. Bacterial itaconate degradation promotes pathogenicity. Nat Chem Biol **10:**371-377.

CHAPTER 3: BUTYRICICOCCUS PORCORUM SP. NOV. A BUTYRATE-PRODUCING BACTERIUM FROM THE SWINE INTESTINAL TRACT

Authors: Julian Trachsel[1,2], Samuel Humphrey[1], and Heather K. Allen[1]*

Author affiliations:

1 Food Safety and Enteric Pathogens Research Unit, National Animal Disease Center, Agricultural Research Service, Ames, IA 50010, USA

2 Interdepartmental Microbiology Graduate Program, Iowa State University, Ames, IA, 50010, USA

Abstract

A Gram-positive, non-motile, butyrate-producing coccus was cultured from the distal ileum of swine. This organism was isolated on rumen fluid medium, consumes acetate, and produces butyrate as a major end product. A phylogenetic analysis based on full-length 16S rRNA gene sequences as well as whole-genome phylogenies suggests that this isolate is most closely related to species in the genus *Butyricicoccus*, with *B. pullicaecorum* being the closest named relative (93.5% 16S identity). The draft genome is available through GenBank under accession number NHOC00000000. The G+C content of this isolate is 54 mol%, and the major cellular fatty acids are $C_{16:0}$, $C_{18:0}$, $C_{14:0}$, $C_{15:0}$-anteiso, and $C_{15:0}$-iso. These data indicate that this isolate represents a novel species within the genus *Butyricicoccus,* for which we propose the name *Butyricicoccus porcorum* sp. nov. The type strain of *Butyricicoccus porcorum* is BB10^T (ATCC XXXXX^T, DSM 104997^T).

Introduction

The family *Ruminococcaceae* harbors many important intestinal commensal organisms, though study of this family has lagged behind other intestinal commensal families such as the *Lachnospiraceae* (based on the number of public genome assemblies, 188 vs. 427, respectively). Members of the *Ruminococcaceae* family participate in a diverse array of functions, and many of its members are butyrate producers. Butyrate is a compound excreted by bacteria as they ferment dietary fiber in the large intestine and is necessary for maintaining intestinal homeostasis and resistance to certain enteric pathogens (1-10). As a result, having cultured representatives of butyrate-producing bacteria is important to study their interactions with the host, diet, and pathogens. The genus *Butyricicoccus* is within the family *Ruminococcaceae,* and several members of this genus have been isolated (11-19), yet most remain uncharacterized. One study has shown that members of this genus are mucosa-associated and are reduced in patients with ulcerative colitis (20). Members of this phylogenetic group, such as *B. pullicaecorum*, can have beneficial impacts on the host, including a reduction in the severity of 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis (21) and more recently some beneficial outcomes for poultry in a model of necrotic enteritis (22). Several *Butyricicocci* have been isolated from the swine intestinal tract, yet they are all unique isolates based on 16S rRNA sequence identity and phylogenetic placement. Here we describe strain BB10, an isolate from the swine intestinal tract, and suggest that it represents a novel species of the genus *Butyricicoccus*. We propose the name *Butyricicoccus porcorum* sp. nov. for this species.

The taxonomy of the clade containing the *Butyricicocci* is currently conflicted. For example, two research groups have each suggested a different taxonomic fate for the species *Eubacterium desmolans*. Takada et al. have suggested that *E. desmolans,* along with their novel

isolate, *B. faecalihominis* KS-2, be classified into the existing *Butyricicoccus* genus (13). In contrast, Ahn et al. have suggested that *E. desmolans,* along with their isolate *A. butyriciproducens* SR79, be classified into the proposed genus *Agathobaculum* (12). These two strains (*B. faecalihominis* KS-2 and *A. butyriciproducens* SR79) appear to be members of the same species as their 16S genes are 99% identical; however, neither genome sequence is publicly available, impeding additional comparisons. This inconsistency in strain classification, in addition to the lack of characterization of strains from this phylogenetic neighborhood, show that a comprehensive comparison of all *Butyricicoccus*-related strains is needed to clarify the taxonomic boundaries of this genus.

Isolation

Strain BB10 was isolated from a 3-month-old pig fed a standard corn-based diet without recent exposure to antibiotics. All bacterial manipulations were performed in a Coy anaerobic chamber (86% N_2, 10% CO_2, 4% H_2). At necropsy, mucosal scrapings were collected, homogenized in sterile anaerobic phosphate buffered saline, and serially diluted. Dilutions of the homogenates were plated on rumen-fluid agar medium (RTY; supplement). Plates were incubated anaerobically at 39°C for 2 days, then colonies were picked and re-streaked for purity. Isolates with unique morphologies were subjected to colony PCR and 16S rRNA gene sequencing with the primers 8F and 1492R. Analysis of the sequences using the Ribosomal Database Project's online tools (23) revealed strain BB10 as a novel bacterial species based on sequence identity to known species (~93% identity to *B. pullicaecorum*).

Growth characteristics

Strain BB10 grew well in RTY medium (Supplement) at pH 6.0. A defined medium was designed to test utilization of distinct carbohydrates and amino acids, and all subsequent characterizations were carried out in this defined medium either with or without acetate (BB10 characteriation media, supplement). Optical densities at 600nm were measured using a BioReaderC plate reader in an anaerobic chamber. We next measured short chain fatty acid (SCFA) production by GC analysis under various growth conditions as described (24). BB10 was unable to use complex carbohydrates and only showed robust growth ($OD_{600} > 0.7$) on mono- or di-saccharides. Butyrate, CO_2, and H_2 were produced and acetate was consumed when grown on glucose, fructose, galactose, sucrose, maltose, or lactose. Moderate growth ($OD_{600} > 0.5$) was observed on myo-inositol. Weak growth ($OD_{600} < 0.4$) was observed when grown on raffinose. No growth ($OD_{600} < 0.1$) was detected when grown on trehalose, mannitol, mannose, glycerol, melezitose, sorbitol, rhamnose, n-acetylglucosamine, n-acetylgalactosamine, arabinose, cellobiose, fucose, xylose, inulin, pectin, potato starch, or mucin. In addition, strain BB10 was unable to use any free amino acids as a carbon source. These results suggest that BB10 requires simple carbohydrates and lacks the ability to metabolize complex substrates in isolation.

Morphology

Phase-contrast microscopy revealed that cells of BB10 cultured in RTY medium were non-motile. Cells of BB10 were prepared for transmission electron microscopy from stationary phase cultures (48 hours), and were negatively stained with osmium and then visualized under a Tecnai 12 G^2 Biotwin microscope for measurements. Cells of BB10 were cocci and were approximately 1.5µm in diameter, and they occurred singly, in pairs, and in tetrads during the stationary phase (Figure S1A). During the early growth phase the cells appeared as irregular agglomerations of larger spherical protrusions (Figure S1B). Cells showed an affinity to clump

together as if they were bound by an extracellular matrix. In liquid culture, cells of BB10 clumped together in a mucoid mat (Figure S2). TEM micrographs revealed a capsule

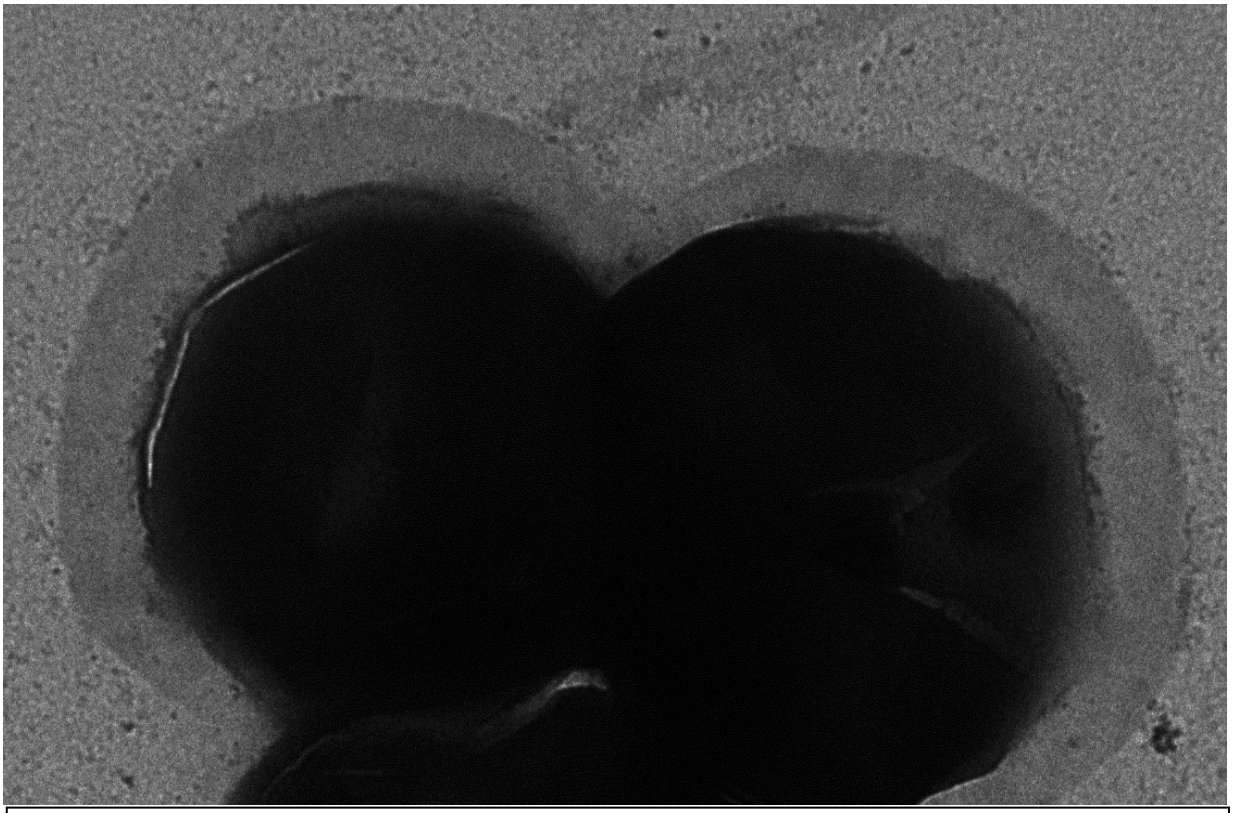

Figure 1: A TEM micrograph of BB10 showing cells surrounded by a polysaccharide capsule

surrounding the cell wall (Figure 1).

Genomic analysis and comparison with other *Butyricicocci*

All available genomes for members of the putative *Butyricicoccus* clade of the *Ruminococcaceae* family were uploaded to RAST (25) for annotation, metabolic prediction, and comparison with BB10 (Genbank accession NHOC00000000). BB10 contained many genes that putatively encode exopolysaccharide biosynthesis and modification of the capsule with sialic acid. This supports our observation of an apparent extracellular capsule (Figure 1) and of mucoid aggregates in liquid culture (Figure S2). The ability to secrete exopolysaccharides has

important implications for host health in another well-known member of the *Ruminococcaceae*, *Faecalibacterium prauznitzii*. Rossi et. al. demonstrated that a strain of *F. prauznitzii* secreted an exopolysaccharide matrix and attenuated dextran sodium sulphate (DSS) induced colitis better than one that did not produce exopolysaccharides (26). All of the other members of the putative *Butyricicoccus* clade lacked the 'exopolysaccharide biosynthesis SEED subsystem' as determined by RAST.

Several genomic predictions were shared by several putative *Butyricicocci*. All members were predicted to be butyrate producers. In accordance with our previous genomic investigation (27), the complete butyrate synthesis pathway was present in BB10 and was predicted to terminate with the *but* gene. The genome also revealed the absence of the ability to degrade complex polysaccharides, which is in agreement with the culture-based assay results. Indeed, most of the cultured and genome-sequenced representatives of this clade lack the ability to degrade complex substrates. For example, Takada et al. report that strain *B. faecihominis* was isolated from a mucin enrichment culture, yet this bacterium lacks the genes required to degrade the complex mucin molecule on its own. The authors suggest that *B. faecihominis* grows on the degradation products from other mucin-degrading bacteria. Additional examples are the uncultured putative *Butyricicoccus* members that do not encode glycoside hydrolases and relatively few carbohydrate transporters; these organisms were described as "*Clostridial* scavengers" (28). In contrast to BB10, *E. desmolans*, and *B. faecihominis*, some putative *Butyricicocci*, including both *A. butyriciproducens* and *B. pullicaecorum*, were reported to hydrolyze complex carbohydrates (starch)(11, 12).

Not all genomic-based predictions were supported by our phenotypic observations. Many genes in BB10 were annotated as encoding spore formation, but sporulation was not

observed in the growth conditions tested. Similarly, the genomes of all members of this clade contained many genes annotated as encoding spore formation, yet no described isolates of this clade have been observed to form spores. Additionally, the genome annotation of BB10 suggests genes encoding mannose and mannitol utilization; however, growth was not detected on these substrates.

GC content was calculated from genome sequences using a BioPython script (29), or taken from previous publications when available (Table 1). The average GC content of this clade is 54.7 mol%, the minimum is 52.9 mol% (*A. butyriciproducens*), the maximum is 56.9 mol% (*Butyriciccoci*. spp. 2789STDY5834927), with a standard deviation of 1.1 mol% considering all currently available data.

Table 1: The various strains identified to belong to clade containing described *Butyricicoccus* species

Strain	Host	%16S ID to BB10	GC content	reference
B. porcorum BB10	Pig	100	54	-
B. pullicaecorum 25-3	Chicken	93	54.5	14
B. 2789STDY5834927	Human	92	56.9	25
B. sp. N54.MGS-46	Human	92	55	2
Clostridiales bacterium DJF-B152	Pig	93	-	19
butyrate producing bacterium A2-207	Human	92	-	21
Agathobaculum butyriciproducens SR79	Human	93	52.9	15
B. sp. K4410.MGS-46	Human	92	55.6	2
bacterium NLAE-zl-C313	Cow	94	-	18
Clostridiales bacterium 7-4c	chicken	91	-	22
B. faecihominis KS-2	human	92	55.6	16
bacterium NLAE-zl-C312	cow	93	-	18
bacterium NLAE-zl-H60	Human	93	-	-
bacterium NLAE-zl-H55	Human	93	-	-
Eubacterium Desmolans ATCC 43058	Cat	91	54.3	20
bacterium NLAE-zl-H41	Human	93	-	-
bacterium NLAE-zl-C141	Cow	92	-	18
Clostridiales bacterium DJF-CP67	Pig	92	-	19
B. 2789STDY5834926	Human	94	53.7	25

Phylogenetic Analysis

The genetic relatedness of strain BB10 to known bacterial species was evaluated by phylogenetic analysis. Full-length 16S rRNA gene sequences from defined members of the family *Ruminococcaceae* were downloaded from Genbank (12, 13, 30), and those classified as *Butyricicoccus* were downloaded from RDP rather than NCBI because the NCBI taxonomy grouped several putative *Butyricicocci* in the genus *Eubacterium* (23). Publicly available genome sequences for organisms closely related to members of the *Butyricicoccus* genus were downloaded, including several uncultured or unclassified putative *Butyricicoccus* members (28, 31). When appropriate, the 16S rRNA gene sequences were extracted with RNAmmer (32). All 16S rRNA gene sequences were aligned to the SILVA reference alignment (33) using mothur (34), and then a maximum-likelihood tree was generated with RAxML including bootstrap support values (Figure 2) (35). Code for this analysis is available at https://github.com/Jtrachsel/BB10-IJSEM. BB10 clustered with members of the genus *Butyricicoccus* and this clade was separate from the other clades within the *Ruminococcaceae* family. This analysis suggested that strain BB10's closest cultured relative is an unnamed bacterial species isolated from the intestinal tract of a pig in Denmark, *Clostridales* bacterium DJF-B152 (16). A genome-based phylogenetic analysis on the previously mentioned publicly available genomes was conducted in PhyloPhlan (36) to confirm the 16S rRNA gene sequence-based phylogeny. The genome-based phylogeny was congruent with the 16S rRNA gene sequence phylogeny, supporting the assignment of strain BB10 to the *Butyricicoccus* genus within the *Ruminococcaceae* family (Figure S3).

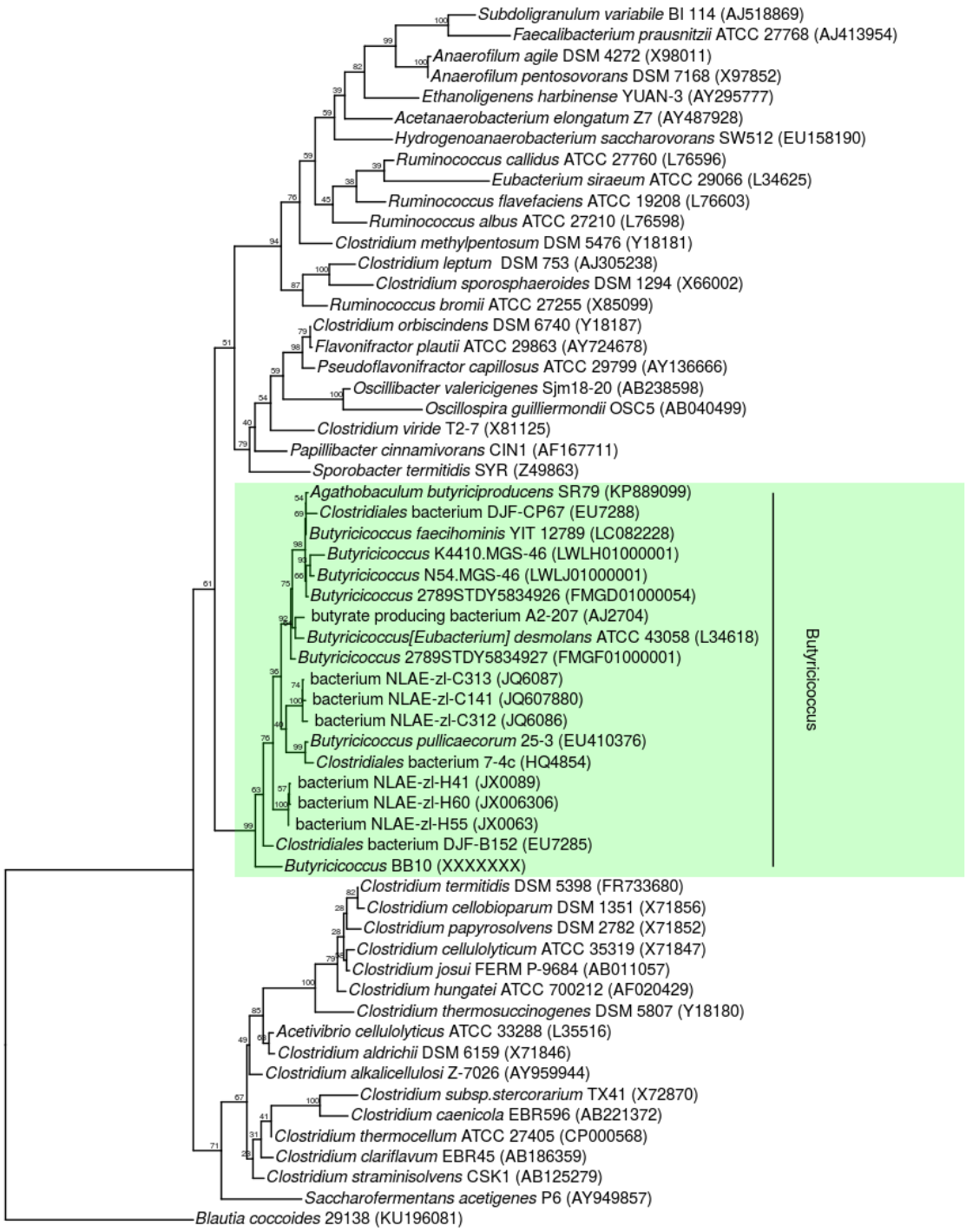

Figure 2: A maximum likelihood tree of full-length 16S rRNA gene sequences from closely related *Ruminococcaceae* family members. *Blautia coccoides* is provided as an outgroup. Bootstrap values are shown at the nodes and represent 1000 samplings.

Host range

All cultured putative members of this clade have been isolated from the intestinal tracts of animals (Table 1). A study by Ziemer et. al. isolated several putative members of this genus from enrichment cultures of bovine and human feces on complex polysaccharides (15). Eekhaut et. al. have isolated members of this clade from the intestinal tracts of chickens (19, 30), and several studies have isolated members of this clade from human feces (12, 13, 18, 31). Full-length 16S rRNA gene sequences nearly identical (99.7%) to that of BB10 have been detected in the feces of pigs in Japan (37) and Canada (38), but have not been reported in other animals. Finally, in our recent functional gene-based study on the butyrate-producing community in the swine proximal colon, we identified many partial butyrate transferase-encoding (*but*) gene sequences that were 100% identical to BB10's *but* gene, suggesting the presence of bacteria very similar to BB10 in the colons of these pigs (27). Taken together, these data suggest that BB10 is a characteristic member of the intestinal microbiota of swine, and that members of this phylogenetic clade are typical members of the intestinal microbiota of many animal species.

Proposed ecological niche of BB10

The biochemical and genomic description of BB10 provides some initial insight into its ecological niche in the gut. Because of the lack of enzymatic machinery or genes to utilize complex substrates in isolation, it is likely that BB10 fits the description of 'Clostridial scavenger' as described in (28). Additionally, it was isolated from the mucosa, and it can metabolize host-derived sugars such as galactose, and so it is likely a butyrate-producing mucosae-associated scavenger. Other members of the *Butyricicoccus* genus have been found to be strongly associated with the mucosa as well (39). Robust occupation of this niche can be beneficial for host health and colonization resistance in a number of ways. First, butyrate

production is well known to improve mucosa health and enhance resistance to colonization by pathogens (5). Secondly, a robust population of scavengers at the mucosa can quickly utilize available simple substrates, thereby depriving pathogenic organisms of access to their preferred food sources (40).

Probiotic potential

BB10 has many features that suggest it may be an attractive probiotic candidate; however, its use as a probiotic faces serious obstacles. Butyrate-producing bacteria are increasingly being considered for probiotic administration and some advances have been made, even within the genus *Butyricicoccus* (30, 41-44). However, like BB10, many butyrate-producing bacteria are strict anaerobes, and this poses some special problems for their use as conventional probiotics. Any exposure of BB10 to oxygen in our experiments quickly resulted in cell death, making conventional probiotic administration techniques problematic. Additionally, even under anaerobic conditions, we observed significant autolysis and loss of viability after 5 days. This loss of viability was observed in both liquid and agar-based culture conditions in a variety of media formulations. Therefore, although BB10 likely has beneficial impacts on the host due to butyrate-production and its role as a potential mucosal scavenger, its applications as a probiotic are currently limited.

Future work may contribute to overcoming the challenges associated with developing BB10 as a probiotic. Most of the obstacles for utility as a probiotic that we identified stem from BB10's apparent fragility and inability to survive long-term outside of the GI tract. However, because BB10 is a commonly occurring member in swine gut microbiota worldwide, it must harbor some mechanisms that aid its dispersion and transmission between hosts. Sporulation is the most obvious mechanism that could achieve this (45), and indeed we detected many genes

thought to be important for sporulation in BB10's genome. Although we were not able to observe spore formation in our studies, it may be that we did not have the correct culture conditions to stimulate spore formation or germination. Future investigations into this bacterium may yield data that allow its use as an effective probiotic organism.

Description of *Butyricicoccus porcorum* sp. nov.

Butyricicoccus porcorum (por.co'rum. L. n. *porcus* swine, pig; L. masc pl. n. *porcorum* of/from pigs).

Cells are obligately anaerobic, non-motile, and coccus shaped. Cells ferment simple mono- and disaccharides. Products of fermentation are butyrate, H_2 and CO_2, dependent on a consumption of acetate. Robust growth is obtained in rumen-fluid media or defined media supplemented with acetate. Optimal growth occurs at 39°C and pH 6.0. After 2 days of growth on agar, colonies are white to off white, ~2 mm in diameter, convex and smooth. In liquid culture this strain aggregates into a mucoid mat. The main cellular fatty acids are $C_{16:0}$, $C_{18:0}$, $C_{14:0}$, $C_{15:0}$-anteiso, and $C_{15:0}$-iso. This strain is a common inhabitant of the swine intestine.

The type strain, BB10^T (ATCC XXXXX, DSM 104997^T), was isolated from the distal ileum of a pig in Ames, Iowa, USA. The DNA G+C content of the type strain is 55 mol%.

Funding Information

This work was supported by the USDA-Agricultural Research Service. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

Acknowledgements

The authors thank Thad Stanton and Torey Looft for scientific advice. We appreciate Judi Stasko for her microscopy services and Nancy Cornick for comments on the manuscript.

Conflicts of Interest

No conflicts of interest.

Ethical statement

Animals were raised in accordance with National Animal Disease Center Animal Care and Use Committee guidelines.

Supplemental Material

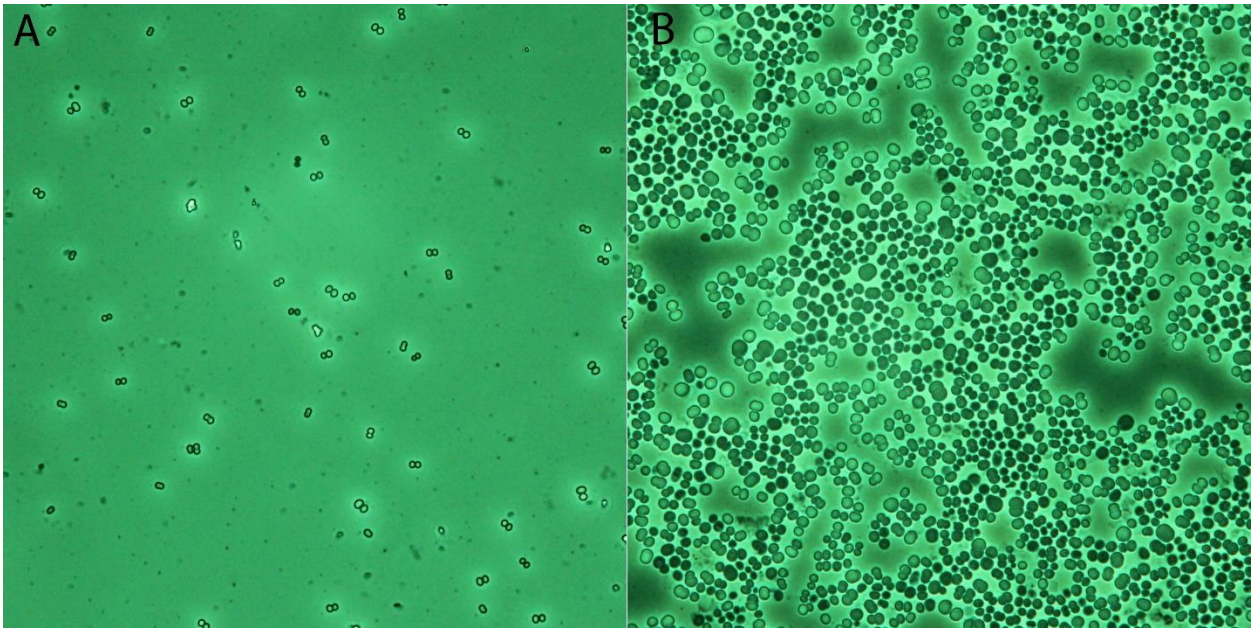

Figure S1: Phase contrast light micrographs of wet mounted BB10 cells at 640x magnification A: After 48 hours of growth on solid agar media. B: After 24 hours of growth on solid agar media

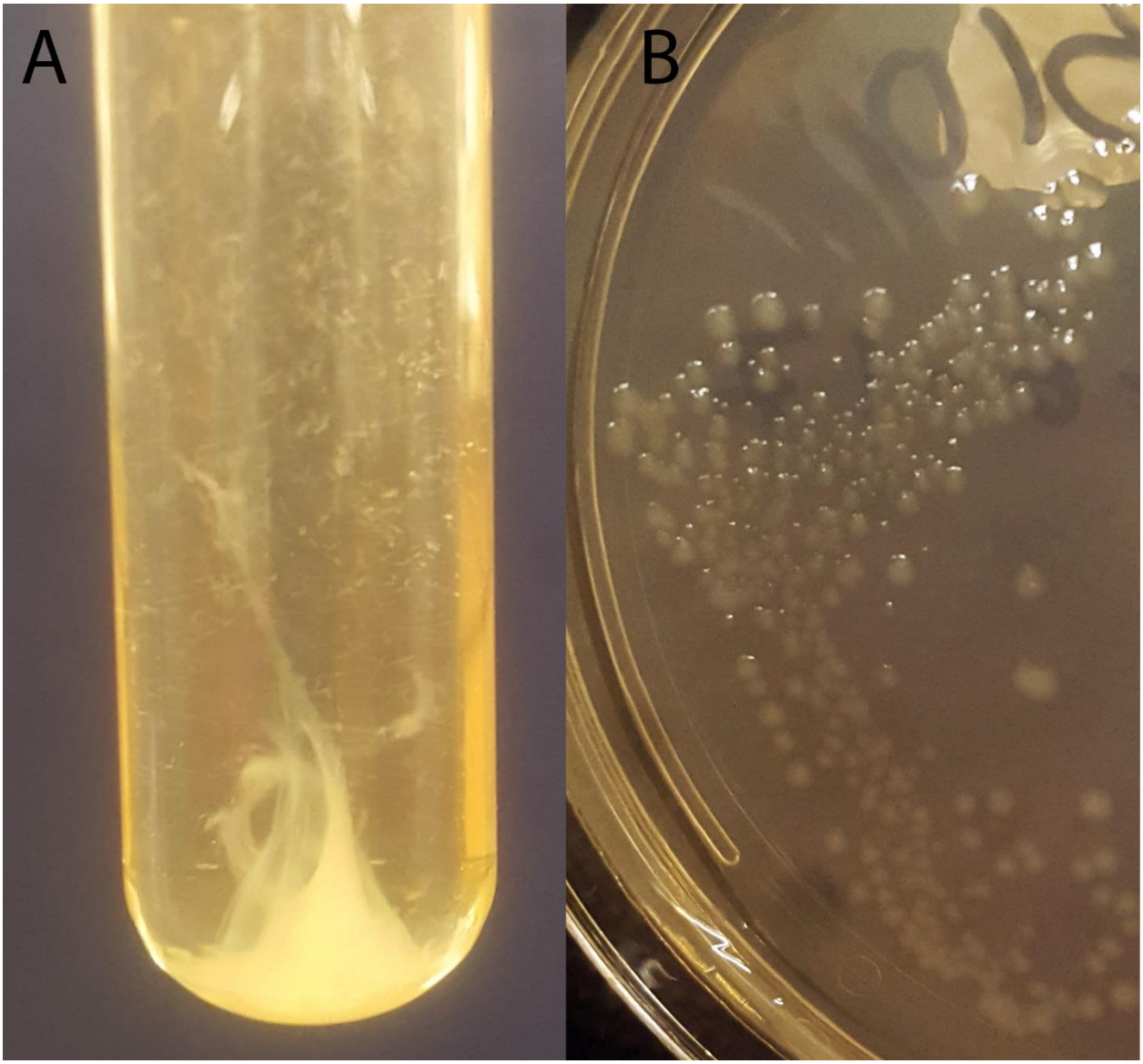

Figure S2: BB10 morphology after 48 hours of growth in A: liquid BB10 general growth medium, B: BB10 general growth agar medium

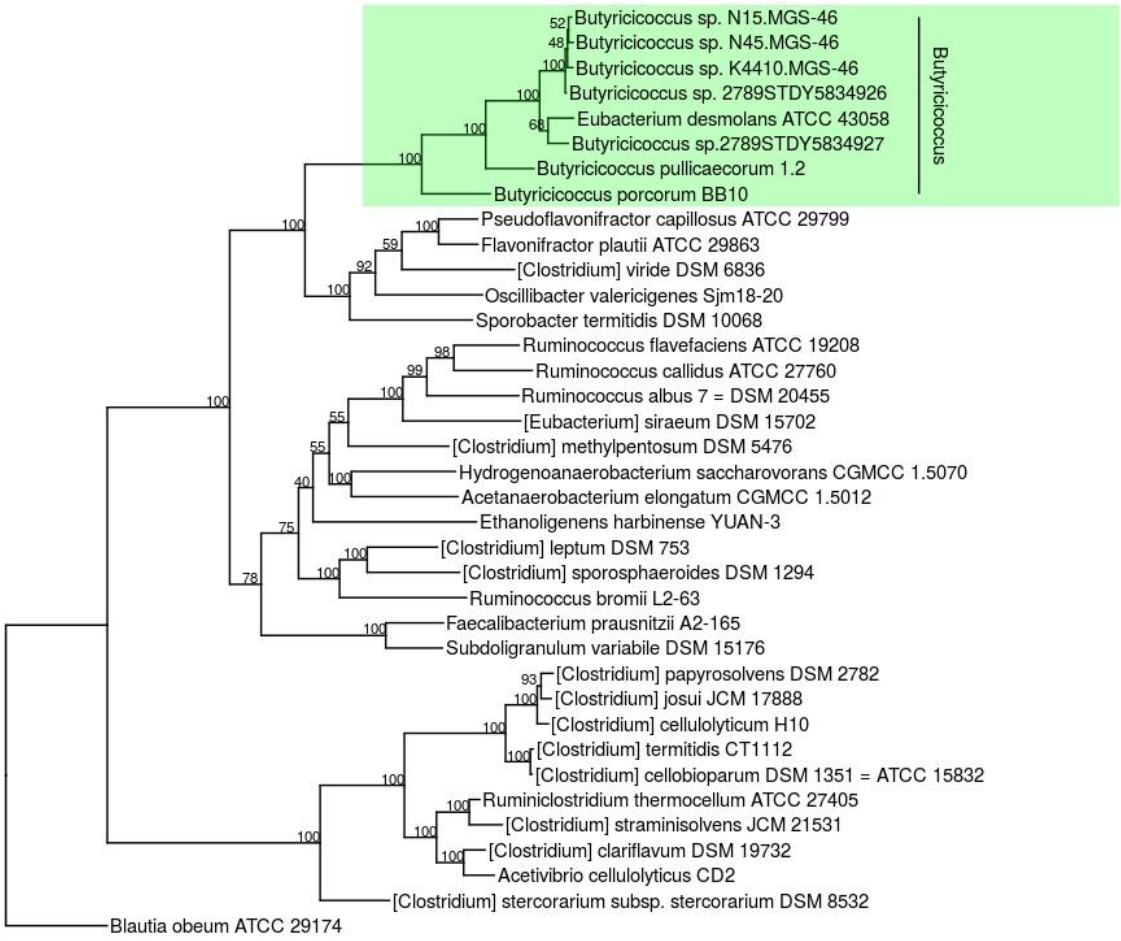

Figure S3: A maximum likelihood tree with bootstrap support values; generated from the concatenated protein alignment output by Phylophlan. Only organisms with publicly available genome assemblies are included here. *Blautia obeum* ATCC 29174 is provided as an outgroup.

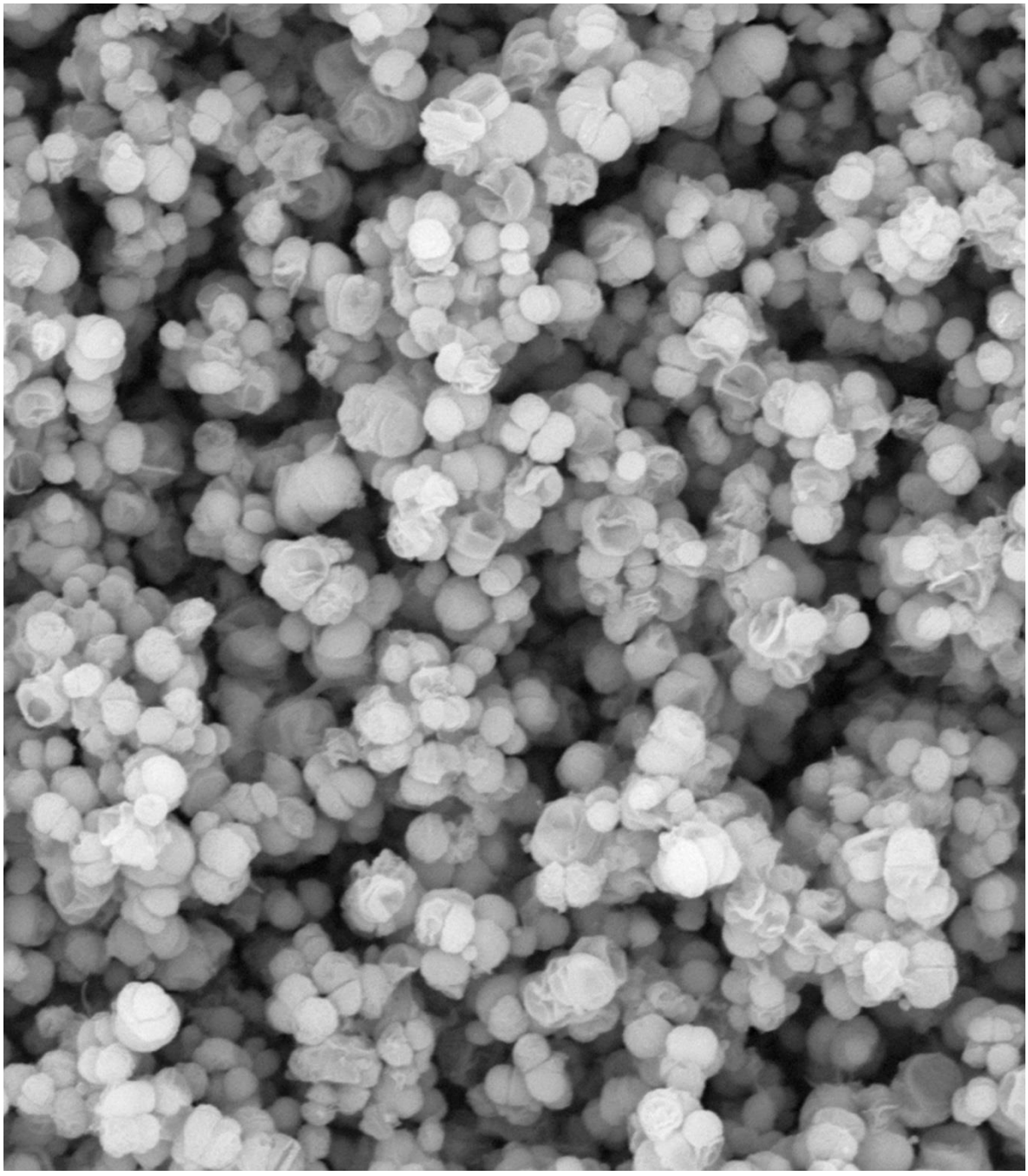

Figure S4: An SEM of BB10 cells after 36 hours of growth

BB10 general growth media (per liter):

Tryptone peptone	5g
Yeast extract	5g
Resazurin (0.1% w/vol)	1mL
Salt solution 1[A]	40 mL
Salt solution 2[B]	40 mL
Hemin solution[C]	1 mL
Vitamin K working stock[D]	1 mL
Cysteine HCl	0.5 g
Glucose	2.5 g
Galactose	2.5 g
Lactose	2.5 g
Maltose	2.5 g
Sodium Acetate	4.5g
dH_2O up to 1 L	

BB10 Characterization media (per liter):

Tryptone peptone	5g
Yeast extract	2.5g
Resazurin (0.1% w/vol)	1mL
Salt solution 1[A]	40 mL
Salt solution 2[B]	40 mL
Hemin solution[C]	1 mL
Vitamin K working stock[D]	1 mL
Cysteine HCl	0.5 g
Add carbohydrate of interest at 1% w/vol	
Sodium Acetate	4.5g
dH_2O up to 1 L	

RTY-rumen fluid (per liter):

Tryptone peptone	5g
Yeast extract	2.5g
Resazurin (0.1% w/vol)	1mL
Salt solution 1[A]	40 mL
Salt solution 2[B]	40 mL
Hemin solution[C]	1 mL
Vitamin K working stock[D]	1 mL
Cysteine HCl	0.5 g
Glucose	10g
Sodium Acetate	4.5g
Clarified Rumen fluid	300 mL
dH_2O up to 1 L	

[A]Salt solution 1 (per liter):

K_2HPO_4	6 g

[B]Salt solution 2 (per liter):

KH_2PO_4 6 g
$(NH_4)_2SO_4$ 12 g
NaCl 12 g
$MgSO_4 \cdot 7H_2O$ 1.2 g
$CaCl \cdot 6H_2O$ 1.2 g

[C]Hemin solution:

0.5g Hemin into 10 mL 1M NaOH. Bring up to 100 mL in dH_2O. Store at 4 C.

[D]Vitamin K solution:

Stock: 0.02 mL vitamin K into 2 mL 95% ethanol. Store in dark at 4 C.
Working stock: add 1 mL stock to 9 mL d H_2O. Use within 3 days.

Cultivation

Cells of BB10 are strictly anaerobic, any exposure to oxygen results in non-viability. We routinely grew BB10 in media using resazurin as a redox indicator. Any media that was slightly pink was unable to support growth. All manipulations were performed in a Coy anaerobic chamber with an atmosphere of 86% N_2, 10% CO_2, 4% H_2. For long term storage of BB10 cells, glycerol freezer stocks were found to be most effective. Briefly, we inoculated liquid BB10 general growth medium and allow cells to grow for 24-48 hours. Ensuring the cells were evenly distributed throughout the culture, we added 500uL cells to 1.8mL screw-top cryovials, then added 500uL BB10 general growth media containing 20% glycerol. We sealed the cryovials in the anaerobic chamber, then froze at -80 C.

Citations

1. Rivera-Chavez F, Zhang LF, Faber F, Lopez CA, Byndloss MX, Olsan EE, Xu G, Velazquez EM, Lebrilla CB, Winter SE, Baumler AJ. 2016. Depletion of butyrate-producing *Clostridia* from the gut microbiota drives an aerobic luminal expansion of *Salmonella*. Cell Host Microbe 19:443-54.
2. Xiong H, Guo B, Gan Z, Song D, Lu Z, Yi H, Wu Y, Wang Y, Du H. 2016. Butyrate upregulates endogenous host defense peptides to enhance disease resistance in piglets via histone deacetylase inhibition. Sci Rep 6:27070.
3. Schilderink R, Verseijden C, Seppen J, Muncan V, van den Brink GR, Lambers TT, van Tol EA, de Jonge WJ. 2016. The SCFA butyrate stimulates the epithelial production of retinoic acid via inhibition of epithelial HDAC. Am J Physiol Gastrointest Liver Physiol 310:G1138-46.
4. Ji J, Shu D, Zheng M, Wang J, Luo C, Wang Y, Guo F, Zou X, Lv X, Li Y, Liu T, Qu H. 2016. Microbial metabolite butyrate facilitates M2 macrophage polarization and function. Scientific Reports 6:24838.
5. Cushing K, Alvarado DM, Ciorba MA. 2015. Butyrate and Mucosal Inflammation: New Scientific Evidence Supports Clinical Observation. Clinical Translational Gastroenterology 6:e108.
6. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, Thangaraju M, Prasad PD, Manicassamy S, Munn DH, Lee JR, Offermanns S, Ganapathy V. 2014. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity 40:128-39.
7. Chang PV, Hao L, Offermanns S, Medzhitov R. 2014. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proceedings of the National Academy of Sciences U S A 111:2247-52.
8. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, Ohno H. 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature 504:446-50.
9. Zimmerman MA, Singh N, Martin PM, Thangaraju M, Ganapathy V, Waller JL, Shi H, Robertson KD, Munn DH, Liu K. 2012. Butyrate suppresses colonic inflammation through HDAC1-dependent Fas upregulation and Fas-mediated apoptosis of T cells. Am J Physiol Gastrointestinal Liver Physiology 302:G1405-15.
10. Fontenelle B, Gilbert KM. 2012. n-Butyrate anergized effector CD4+ T cells independent of regulatory T cell generation or activity. Scandinavian Journal of Immunology 76:457-63.
11. Eeckhaut V, Van Immerseel F, Teirlynck E, Pasmans F, Fievez V, Snauwaert C, Haesebrouck F, Ducatelle R, Louis P, Vandamme P. 2008. Butyricicoccus pullicaecorum gen. nov., sp. nov., an anaerobic, butyrate-producing bacterium isolated from the caecal content of a broiler chicken. International Journal of Systemics and Evolutionary Microbiology 58:2799-802.
12. Ahn S, Jin TE, Chang DH, Rhee MS, Kim HJ, Lee SJ, Park DS, Kim BC. 2016. *Agathobaculum butyriciproducens* gen. nov. sp. nov., a strict anaerobic, butyrate-producing gut bacterium isolated from human faeces and reclassification of *Eubacterium desmolans* as *Agathobaculum desmolans* comb. nov. International Journal of Systemics and Evolutionary Microbiology 66:3656-3661.
13. Takada T, Watanabe K, Makino H, Kushiro A. 2016. Reclassification of Eubacterium desmolans as *Butyricicoccus desmolans* comb. nov., and description of *Butyricicoccus faecihominis* sp. nov., a butyrate-producing bacterium from human faeces. International Journal of Systemics and Evolutionary Microbiology 66:4125-4131.

14. Li X, Hojberg O, Canibe N, Jensen BB. 2016. Phylogenetic diversity of cultivable butyrate-producing bacteria from pig gut content and feces. Journal of Animal Science 94:377-381.
15. Ziemer CJ. 2014. Newly cultured bacteria with broad diversity isolated from eight-week continuous culture enrichments of cow feces on complex polysaccharides. Appl Environ Microbiol 80:574-85.
16. Li X, Hoberg O, Canibe N, Jensen BB. 2016. Phylogenetic Diversity of Cultivable Butyrate-producing Bacteria from Pig Gut Content and Feces. Journal of Animal Science 94:377-381.
17. Morris GN, Winter J, Cato EP, Ritchie AE, Bokkenheuser VD. 1986. Eubacterium desmolans sp. nov., a Steroid Desmolase-Producing Species from Cat Fecal Flora. International Journal of Systematic and Evolutionary Microbiology 36:183-186.
18. Barcenilla A, Pryde SE, Martin JC, Duncan SH, Stewart CS, Henderson C, Flint HJ. 2000. Phylogenetic relationships of butyrate-producing bacteria from the human gut. Applied and Environmental Microbiology 66:1654-61.
19. Eeckhaut V, Van Immerseel F, Croubels S, De Baere S, Haesebrouck F, Ducatelle R, Louis P, Vandamme P. 2011. Butyrate production in phylogenetically diverse Firmicutes isolated from the chicken caecum. Microbial Biotechnology 4:503-12.
20. Backert S, Hofreuter D. 2013. Molecular methods to investigate adhesion, transmigration, invasion and intracellular survival of the foodborne pathogen Campylobacter jejuni. Journal of Microbiological Methods 95:8-23.
21. Ribet D, Cossart P. 2015. How bacterial pathogens colonize their hosts and invade deeper tissues. Microbes Infect 17:173-83.
22. Pizarro-Cerda J, Cossart P. 2006. Bacterial adhesion and entry into host cells. Cell 124:715-27.
23. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM. 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res 42:D633-42.
24. Salanitro JP, Muirhead PA. 1975. Quantitative Method for the Gas Chromatographic Analysis of Short-Chain Monocarboxylic and Dicarboxylic Acids in Fermentation Media. Applied Microbiology 29:374-381.
25. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, Stevens R. 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Research 42:D206-14.
26. Rossi O, Khan MT, Schwarzer M, Hudcovic T, Srutkova D, Duncan SH, Stolte EH, Kozakova H, Flint HJ, Samsom JN, Harmsen HJ, Wells JM. 2015. Faecalibacterium prausnitzii Strain HTF-F and Its Extracellular Polymeric Matrix Attenuate Clinical Parameters in DSS-Induced Colitis. PLoS One 10:e0123013.
27. Trachsel J, Bayles DO, Looft T, Levine UY, Allen HK. 2016. Function and Phylogeny of Bacterial Butyryl Coenzyme A:Acetate Transferases and Their Diversity in the Proximal Colon of Swine. Applied Environmental Microbiology 82:6788-6798.
28. Jeraldo P, Hernandez A, Nielsen HB, Chen X, White BA, Goldenfeld N, Nelson H, Alhquist D, Boardman L, Chia N. 2016. Capturing One of the Human Gut Microbiome's Most Wanted: Reconstructing the Genome of a Novel Butyrate-Producing, Clostridial Scavenger from Metagenomic Sequence Data. Fronteirs Microbiology 7:783.
29. Cock PJ, Antao T, Chang JT, Chapman BA, Cox CJ, Dalke A, Friedberg I, Hamelryck T, Kauff F, Wilczynski B, de Hoon MJ. 2009. Biopython: freely available Python tools for computational molecular biology and bioinformatics. Bioinformatics 25:1422-3.
30. Geirnaert A, Steyaert A, Eeckhaut V, Debruyne B, Arends JB, Van Immerseel F, Boon N, Van de Wiele T. 2014. Butyricicoccus pullicaecorum, a butyrate producer with probiotic potential, is intrinsically tolerant to stomach and small intestine conditions. Anaerobe 30:70-4.

31. Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, Goulding D, Lawley TD. 2016. Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. Nature doi:10.1038/nature17645.
32. Lagesen K, Hallin P, Rodland EA, Staerfeldt HH, Rognes T, Ussery DW. 2007. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res 35:3100-8.
33. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41:D590-D596.
34. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537-41.
35. Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312-3.
36. Segata N, Börnigen D, Morgan XC, Huttenhower C. 2013. PhyloPhlAn is a new method for improved phylogenetic and taxonomic placement of microbes. Nature Communications 4:2304.
37. Kobayashi Y, Itoh A, Miyawaki K, Koike S, Iwabuchi O, Iimura Y, Kobashi Y, Kawashima T, Wakamatsu J, Hattori A, Murakami H, Morimatsu F, Nakaebisu T, Hishinuma T. 2011. Effect of liquid whey feeding on fecal microbiota of mature and growing pigs. Anim Sci J 82:607-15.
38. Kalmokoff M, Waddington LM, Thomas M, Liang KL, Ma C, Topp E, Dandurand UD, Letellier A, Matias F, Brooks SP. 2011. Continuous feeding of antimicrobial growth promoters to commercial swine during the growing/finishing phase does not modify faecal community erythromycin resistance or community structure. J Appl Microbiol 110:1414-25.
39. Devriese S, Eeckhaut V, Geirnaert A, Van den Bossche L, Hindryckx P, Van de Wiele T, Van Immerseel F, Ducatelle R, De Vos M, Laukens D. 2017. Reduced Mucosa-associated Butyricicoccus Activity in Patients with Ulcerative Colitis Correlates with Aberrant Claudin-1 Expression. J Crohns Colitis 11:229-236.
40. Ferreyra JA, Ng KM, Sonnenburg JL. 2014. The Enteric Two-Step: nutritional strategies of bacterial pathogens within the gut. Cell Microbiol 16:993-1003.
41. Eeckhaut V, Wang J, Van Parys A, Haesebrouck F, Joossens M, Falony G, Raes J, Ducatelle R, Van Immerseel F. 2016. The Probiotic Butyricicoccus pullicaecorum Reduces Feed Conversion and Protects from Potentially Harmful Intestinal Microorganisms and Necrotic Enteritis in Broilers. Front Microbiol 7:1416.
42. Geirnaert A, Wang J, Tinck M, Steyaert A, Van den Abbeele P, Eeckhaut V, Vilchez-Vargas R, Falony G, Laukens D, De Vos M, Van Immerseel F, Raes J, Boon N, Van de Wiele T. 2015. Interindividual differences in response to treatment with butyrate-producing Butyricicoccus pullicaecorum 25-3T studied in an in vitro gut model. FEMS Microbiol Ecol 91.
43. Eeckhaut V, Ducatelle R, Sas B, Vermeire S, Van Immerseel F. 2014. Progress towards butyrate-producing pharmabiotics: Butyricicoccus pullicaecorum capsule and efficacy in TNBS models in comparison with therapeutics. Gut 63:367.
44. Van Immerseel F, Ducatelle R, De Vos M, Boon N, Van De Wiele T, Verbeke K, Rutgeerts P, Sas B, Louis P, Flint HJ. 2010. Butyric acid-producing anaerobic bacteria as a novel probiotic treatment approach for inflammatory bowel disease. J Med Microbiol 59:141-3.
45. Meehan CJ, Beiko RG. 2014. A phylogenomic view of ecological specialization in the *Lachnospiraceae*, a family of digestive tract-associated bacteria. Genome Biol Evol 6:703-13.

CHAPTER 4: FUELING BENEFICIAL HOST-BACTERIAL INTERACTIONS IN THE SWINE GUT WITH DIETARY RAW POTATO STARCH

Authors: Julian Trachsel, Cassidy Briggs, Nicholas Gabler, Crystal Loving, Heather K. Allen

This work is currently being prepared for submission

Introduction

Weaning is a stressful time in the life of a piglet. In rapid succession, weaned piglets experience a diet change, transport stress, exposure to previously unencountered pathogens, the stress of social integration, and the decline of maternal-derived antibody protections. All of these factors leave the weaned piglet susceptible to infections and complications such as post-weaning diarrhea (1). To combat these weaning-associated diseases, antibiotics are routinely given to nursery-aged pigs. In its 2012 National Animal Health and Monitoring System (NAHMS) report, the USDA reported that almost 90% of nursery sites and almost 98% of wean-to-finish sites administered in-feed antibiotics that year (2). These statistics underscore the need for alternative treatments aimed at supporting the piglet through the post-weaning period. Dietary prebiotics, such as resistant starches, provide an attractive alternative to antibiotics in this regard (1, 3).

Resistant starches are dietary compounds that escape host digestion and improve gut health by delivering microbial-accessible carbohydrates (MACs) to the large intestine. The microbes that consume resistant starches are normal members of the microbiota and are associated with intestinal health (4-8). Recently, it has been shown that without access to diet-derived carbohydrates, commensal bacteria will harvest host-derived sugars from the mucus

layer, degrading and compromising its barrier function (9). However, if ample dietary MACs are present, intestinal microbes will consume them and release beneficial metabolites, such as short chain fatty acids (SCFAs) in the process. Host tissues consume the vast majority of these SCFAs, and in part this fuels the effort to maintain intestinal homeostasis (10, 11). For example, microbiota-derived SCFAs are known to fuel B-cells and enhance their IgA responses (12). While most SCFAs can be used by host tissues, butyrate has been shown to be of special importance for many aspects of gut health.

Butyrate is a central metabolite for maintaining intestinal homeostasis. It is the preferred fuel source for colonocytes which, while oxidizing butyrate, consume considerable amounts of oxygen. This lowers the oxygen potential of the epithelia, reducing the amount of electron acceptors available for microbial respiration and favoring species that use a fermentative metabolism and preventing the overgrowth of facultative anaerobic bacteria, including opportunistic pathogens such as certain *Salmonella* and *E. coli* species. Butyrate also aids in the maintenance of homeostasis by regulating the expressions of many genes in host tissues via histone deacetylase inhibitor activity. Exposure to butyrate and other SCFAs has been shown to increase mucus and antimicrobial peptide secretion (13-16), and shift host immune responses to a more tolerogenic phenotype (17-20). These changes help to limit inflammation and, in doing so, reduce the amount of immune-derived reactive oxygen species or other electron acceptors that can support microbial respiration, further limiting the niche for facultative anaerobes (21, 22).

Resistant starches are a potential feed additive to improve gut health and reduce the need for antibiotic treatment for post-weaning disease susceptibility in pigs. Some pig producers are currently feeding raw potato starch (RPS)to nursery-aged pigs and its use has shown some promising benefits (23). However, the mechanisms by which resistant starch, and specifically

RPS, supports intestinal health in the weaned piglet are poorly defined. Here we investigate how RPS intake can benefit interactions between the intestinal microbiota and the host. The results reported here suggest that dietary resistant starches can help support beneficial host-microbe interactions in the piglet's intestinal ecosystem, increasing resistance to disease by limiting the niche for mucosal pathogens.

Methods

Experimental design

Ten pregnant, Large White crossbred sows were delivered to the National Animal Disease Center (NADC) 2 weeks prior to farrowing and were farrowed onsite. At 14 days-of-age, piglets were offered non-amended Phase 1 starter diet (Table S1) in small bowls placed into each farrowing pen. At 21 days-of-age, piglets were weaned, and separated into 2 treatment groups. Each group consisted of two pens of 7 piglets for a total of 14 piglets in each treatment group. The control group continued to receive non-amended Phase 1 Starter Diet. The treatment (RPS) group was fed Phase 1 Starter Diet amended with 5% raw potato starch (MSP Starch Products Inc., Carberry, Manitoba, Canada). At 33 days-of-age (12 days post-weaning), the control group was switched to Non-amended Phase 2 Diet and the RPS group piglets were switched to Phase 2 Diet amended with 5% raw potato starch (Table S1). At 42 days-of-age (21 days post-weaning), 7 piglets from each group (3 from one pen and 4 from a second pen) were humanely euthanized and various intestinal samples were collected.

Flow Cytometry

Cecal tissues were gently rinsed in PBS to remove digesta. A 2 g section was placed in complete (c) RPMI (RPMI 1640 [Life Technologies; Grand Island, NY] supplemented with 10%

fetal calf serum [FCS, Omega Scientific; Tarzana, CA], L-glutamine [Life Technologies], 25 mM HEPES [Sigma; St. Louis, MO], and essential amino acids and antibiotics [Sigma]) and stored on ice until processing. Protocol has been previously described in (Goodyear et al. 2014). All centrifugation steps were 450 x g for 8 min at 4°C. Tissue was added to 30 mL solution of 5 mM dithiothreitol (DDT) (cat: 15508-013, Invitrogen) and 2% fetal calf serum (FCS) in calcium/magnesium-free Hank's balanced salt solution (HBSS), and incubated at 37°C and agitated at 200 rpm for 20 min. Tissue was transferred to 30 mL epithelial removal solution containing 5 mM EDTA and 2% FCS in HBSS, and incubated for 15 minutes on the shaker as above. This was repeated and the epithelial cells released during these 2 steps were collected by centrifugation. The remaining tissue was transferred into a wash solution containing 10 mM HEPES (Sigma) in HBSS and incubated for 10 min on the shaker as above. Tissue was transferred to a C-tube (Miltenyi Biotec) with 14 mL enzyme digestion media containing 1% HEPES in HBBS, with 0.2 U/mL Liberase TM Research Grade (Roche Life Sciences), and 30 µg/mL DNase I (cat: D5025, Sigma Aldrich), minced with scissors, and processed on gentleMACS Octo Dissociator (Miltenyi Biotec) intestine setting for C-tubes before and after a 45 min incubation on the shaker as above. Debris was removed by filtration through a gauze pad and enzymes were inactivated by addition of 7 mL cRPMI. Cells were run over a 40 µM filter to remove debris, collected by centrifugation, and resuspended in HBSS containing 200 mM L-glutamine and 2% FCS. Epithelial cells released after incubation in EDTA solution were recovered via the same centrifugation and combined with cells from the lamina propria. Approximately 10^6 cells per animal were used for flow cytometric analysis.

Cells were stained with Zombie Yellow Viability dye, followed by incubation with fluorescently-conjugated anti-porcine monoclonal antibodies purchased from BD Biosciences,

San Jose, CA (except as noted), Antibodies used included anti-porcine CD3 (clone BB23-8E6-8C6, cat: 561477), CD4 (clone 74-12-4, cat: 559585), CD8α (clone 76-2-11, cat: 559584ID), CD25 (clone K231.3B2, Southern Biotech cat: 1070-19), and FOXP3 (clone FJK16s, cat: 48-5773-82). A cocktail of all surface marker antibodies was added to each cell suspension, followed by fixation and permeabilization for intracellular staining with anti-Foxp3 antibody using Intracellular Nuclear Staining Kit according to manufacturer's recommendations (Biolegend). Data was acquired on a BD LSRII machine and data was analyzed with FlowJo Software. Representative flow plot is available Figure S1.

RNA extraction, cDNA synthesis, and RT-qPCR of cecal tissue

Cecal mucosal scrapings were stored in RNAlater® (Life Technologies) at 4°C. RNA was extracted using the TriReagent (Life Technologies)-modified protocol of the PowerLyzer UltraClean Tissue & Cells RNA Isolation Kit (MoBio Laboratories, Inc.) following manufacturer's instructions. Homogenization in TriReagent was carried out in a Thermo Savant FastPrep® FP120 Cell Disrupter (Qbiogene, Inc., Carlsbad, CA) with 2 cycles of 45 seconds at 4.0 m/sec and a 30 second pause between cycles. An on-column DNA removal step was included (On-Spin Column DNase I Kit, Mo Bio Laboratories, Inc.). Genomic DNA (gDNA) removal and cDNA synthesis (1 ug), were carried out with QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA).

Recipes for qPCR reactions included 3 µL cDNA, 10 µL TaqMan® Universal Master Mix II (Applied Biosystems, Foster City, CA), with uracil N- glycosylase (UNG) (ThermoFisher), 1 µL Primer/Probe Mix (Life Technologies, Table 2), and 6 µL Nucleic Acid-free water. Cycling conditions were 40 cycles of 95°C for 15 sec and 60°C for 1 minute. The reference gene β-actin was used to normalize the relative expression of genes of interest

according to the $2^{-\Delta\Delta Cq}$ method as described by (Schmittgen and Livak 2008). Genes are described in Table S2.

Microbial community analysis

Feces and cecal contents were collected fresh and stored at -80 C until processing. Additionally, cecal contents as well as mucosal scrapings from two 4 x 6 inch sections were preserved in 5 mL RNAlater® each until RNA extraction. DNA and RNA was extracted using the PowerMag fecal DNA/RNA extraction kit (MoBio). RNA samples were treated with DNase Max (MoBio) kit to remove gDNA and converted to cDNA using the High Capacity cDNA Synthesis Kit (Applied Biosystems). Amplicons of the V4 region were generated and sequenced in accordance with the protocol outlined in Kozich et al. (24). The PCR conditions were: 2 min at 95°C, 22 cycles of [20 sec at 95°C, 15 sec at 55°C, 5 min 72°C], 72°C for 10 min. Libraries were normalized using the SequalPrep Normalization Plate Kit (LifeTechnologies) and quantified using Kapa SYBR Fast qPCR (Kapa Biosystems, Wilmington, MA). Normalized pools were sequenced using version 2 (250x2) chemistry on the MiSeq instrument (Illumina). Amplicons of the *but* gene were generated and sequenced using the protocol described in (25). Briefly, *but* genes were amplified from purified DNA or cDNA using AccuPrime Taq and the funbuts primers. The first-step PCR mixtures contained each primer at 500 nM and 100 ng of template and used an annealing temperature of 45°C for 35 cycles. The second PCR step was performed in accordance with the protocol using Kapa Hifi polymerase (Kapa Biosystems, Wilmington, MA) and the Nextera XT version 2 indices (Illumina, city, state). This library was sequenced on a MiSeq using a 2 x 300 V3 reagent kit (Illumina) to generate 300-bp paired-end reads.

Microbial Glycoside Hydrolase activities

Glycoside hydrolase assays were carried out as in Desai et al. 2016 (9). The following substrates were tested: 4-nitrophenol sulphate, N-acetyl beta-D-glucosamide, beta-D galactopyranoside, alpha-L fucopyranoside, beta-D glucopyranoside, beta-D xylopyranoside, alpha-D galactopyranoside, and alpha-D glucopyranoside. Total proteins were extracted from 200 mg of feces in 1 mL of assay buffer (50 mM Tris, 100 mM KCl, 10 mM $MgCl_2$; TritonX; protease inhibitor; pH 7.2) in a 96 well plate (2 mL deepwell) with 0.1mm beads. Proteins were extracted by bead beating using a Retsch mixing mill (Retsch, Newtown, PA) at 30 cycles/ second for one minute and then placed on ice for 1 minute, repeated 4 times. Debris was pelleted by centrifugation at 4500 x g for 10 minutes at 4 C. Protein concentrations were determined by Pierce BCA assay (Thermoscientific, Rockford, IL) and the same assay buffer was used to normalize protein concentrations. Each assay received 10 ug of protein with a final substrate concentration of 10mM and a final volume of 150 uL in 100 well Bioscreen C plates (Growth Curves USA, Piscataway, NJ). Plates were incubated at 39 C and absorbance at 405nM was measured every 2 minutes using a Bioscreen C plate reader. Rates are reported as mM/s*ug protein.

IgA measurements

Cecal contents (~250 mg) were lyophilized, resuspended in extraction buffer at 30 mg dry weight per mL (recipe here), and then vortexed on high for 10 minutes. Debris was pelleted by centrifugation at 5000 x g and the supernatant was used as an input in the Pig IgA ELISA Quantitation kit (Bethyl Laboratoties, Montgomery, TX). Results are reported as ng IgA/mg dry contents.

Short-chain fatty acid (SCFA) measurements

One gram of material (cecal contents or feces) was resuspended in 2 mL PBS and vortexed for 1 minute, then debris was pelleted by centrifugation at 5000 x g for 10 minutes. Supernatant (1 mL) was added to heptanoic acid internal standards. Butylated fatty acid esters were generated as described in (26) and analyzed using an Agilent 7890 GC (Agilent, Santa Clara, CA).

Data analysis

All R scripts used in this analysis are available at https://github.com/Jtrachsel/RPS-2017. Unless otherwise stated Wilcoxon tests were used to test for statistical differences between groups. Both the 16s rRNA gene sequence and *but* amplicon data were clustered into OTUs in mothur using the Miseq SOP (27). 16S rRNA gene sequences were aligned to the SILVA reference alignment, and *but* sequences were aligned to an alignment of *but* reference sequences downloaded from RDP's fungene database (28).

The R package vegan (29) was used to carry out ecological diversity analyses. For all community data types (16S rRNA gene sequences, *but* amplicons, flow cytometry data) samples were each rarefied to a standard number of observations. Community structure similarity analyses were performed by calculating Bray-Curtis similarities, and statistical testing was accomplished using vegan's Adonis and betadisper functions. Differential abundance was determined using the DeSeq2 package (30).

Correlations for network analysis were calculated with CCREPE (31) for compositional data or the rcorr function from the hmsic R package (32). Network layout and visualization was

done using the geomnet R package (33). Only significant, positive correlations with a Spearman coefficient of at least 0.6 are shown.

Results

Microbial changes through the post-weaning period

We first assessed changes in the microbiota over time after weaning regardless of RPS treatment. Both of the treatment groups experienced similar changes relating to the maturation of their microbiota over the 3 weeks after weaning. The phyla *Proteobacteria, Synergistetes, Fusobacteria, Euryarchaeota,* and *Verrucomicrobia* decreased in abundance while *Bacteroidetes* and *Firmicutes* increased over time after weaning (**Figure 1A**). Additionally, the RPS-fed pigs had an increase in the phylum *Actinobacteria,* driven mainly by an increase in the amount of the genus *Bifidobacteria*. Changes in major phyla were reflected in a large shift in the community structure of the fecal microbiota (**Figure 1B**). The size of this effect was greater than the treatment effects.

The functional capacity for carbohydrate utilization was also examined over time because we hypothesized that due to the diet change at weaning the gut microbiota would have an immature ability to degrade the new dietary substrates. Fecal microbial glycoside hydrolase activities shifted as the gut microbiota matured (**Figure 1C**). Importantly, we observed higher microbial hydrolase activities toward host-derived substrates early. In particular, enzymatic activity to alpha-L fucopyranoside peaked at day 12 post-weaning and decreased as the piglets aged, suggesting that more microbes were sourcing their carbon consumption from host-derived compounds rather than dietary-derived ones early in the post-weaning period. This spike was attenuated in RPS pigs, although the inter-group difference was not significant. In contrast, we

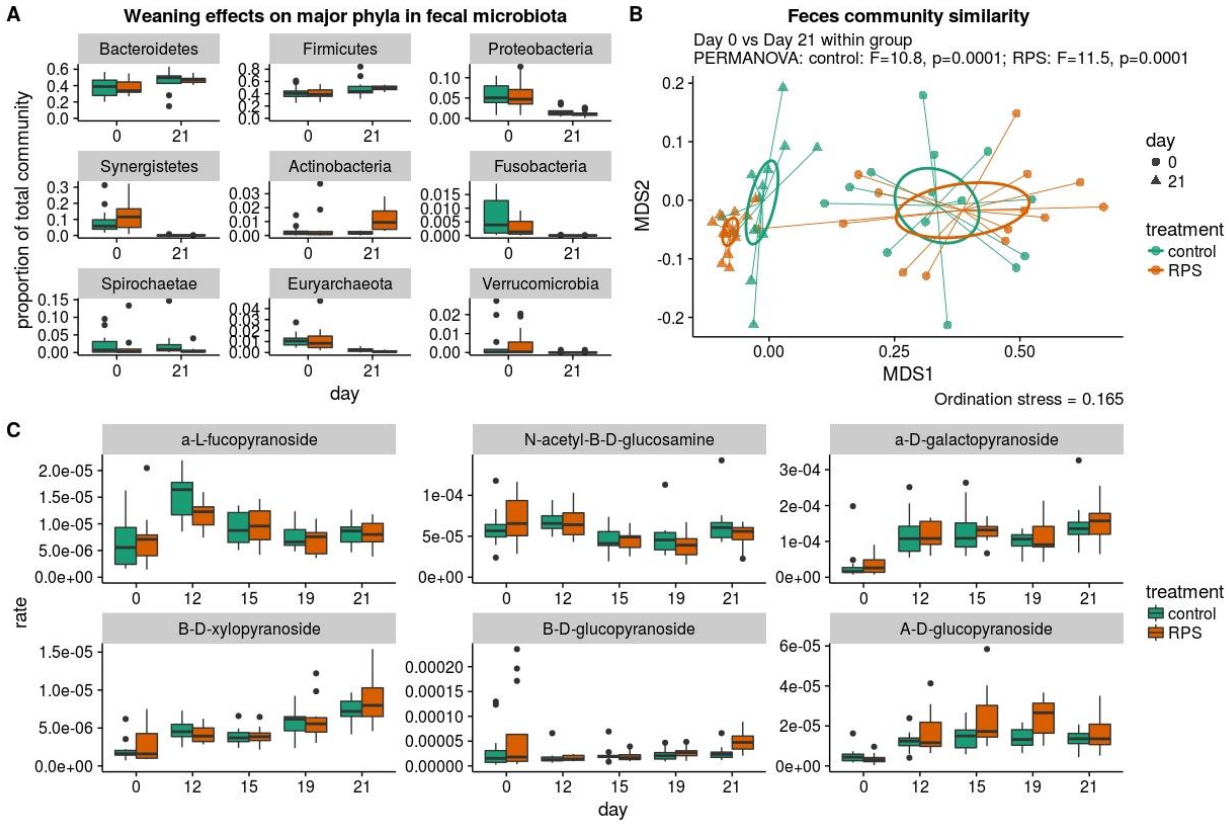

Figure 1: A: Changes in major phyla of the fecal microbiota from day 0 to day 21 post-weaning. **B:** A Bray-Curtis based NMDS ordination depicting fecal microbiota community similarities from day 0 to day 21 post-weaning. Ellipses indicate the standard error of the group **C:** Glycoside hydrolase activities of the fecal microbiota from day 0 to day 21 post-weaning.

observed gradually increasing hydrolase activities on plant-derived substrates throughout the 21 days post-weaning, aligning with an enrichment of members of the microbiota that can metabolize plant glycans. Taken together, the results show that the microbiota of the newly weaned pig shifts from metabolizing host glycans to plant glycans, and this shift is gradual despite the instant shift in diet at weaning. RPS intake did not significantly alter these trajectories, though it tended to increase a-D-glucosidase activity (the activity required to digest RPS), group differences were not significant.

Microbial communities of the treatment groups diverged as they matured

Although the two groups experienced similar weaning-related changes in their microbial communities, the fecal bacterial community structures of the two treatment groups gradually diverged, (**Figure 2A, Table S3**) and by day 21 the two treatment groups had significantly different fecal microbiota community structures. Group differences in community profiles were seen in both the broader 16S rRNA gene sequence-based bacterial community as well as the *but*-based community (one important portion of the butyrate-producing community). The two treatment groups did not have significantly different community structures until Day 15, which corresponded to a decrease in the lactose in the diets, as is common practice in commercial operations. The size of these effects were driven in part by community dispersion **(Figure 2B)**, with the 16S rRNA gene sequence analysis of the communities of the RPS pigs exhibiting significantly less group variability compared to the control pigs at day 21, and both groups became less dispersed as they matured (**Table S4**).

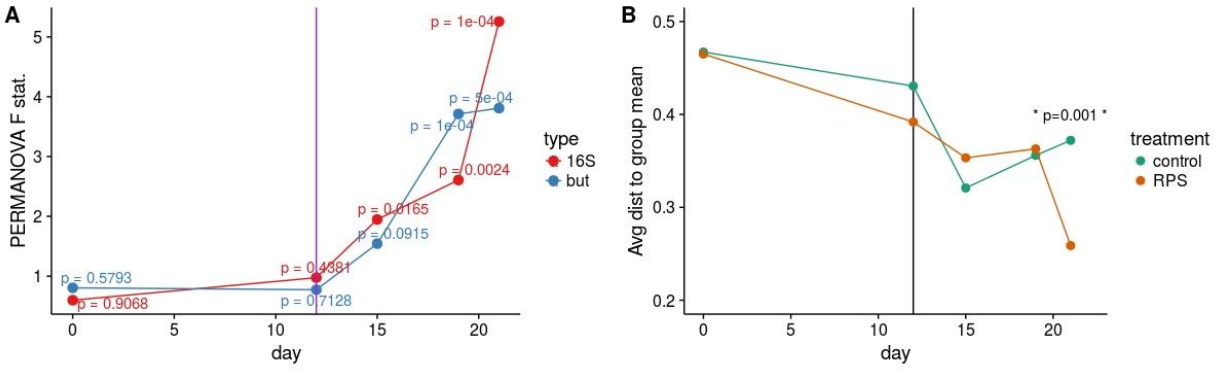

Figure 2: A: PERMANOVA pseudo F statistic over time for both the 16S- and *but*-based fecal microbial communities. This is a measure of how different the control and RPS fecal communities are at each timepoint. P-values are shown at each timepoint. **B:** Group dispersion over time (calculated by the bdisp function from the vegan package).

Twenty-one days after weaning, the structure of the microbiota was significantly different between the treatment groups, and these differences resulted in differing profiles of microbial metabolites. We observed differences in microbial community structure among many tissues, and the 16S rRNA gene sequence-based communities tended to be more different than the butyrate-producing communities (**Figure 3A,B and Table S3)**. This result suggests that changes in bacterial membership are only partially reflected in bacterial functions, which is likely due to the functional redundancy among members within an ecosystem. The tissue-associated microbial communities exhibited the same dispersion trend as the fecal communities, with the RPS communities exhibiting less group dispersion (Table S3).

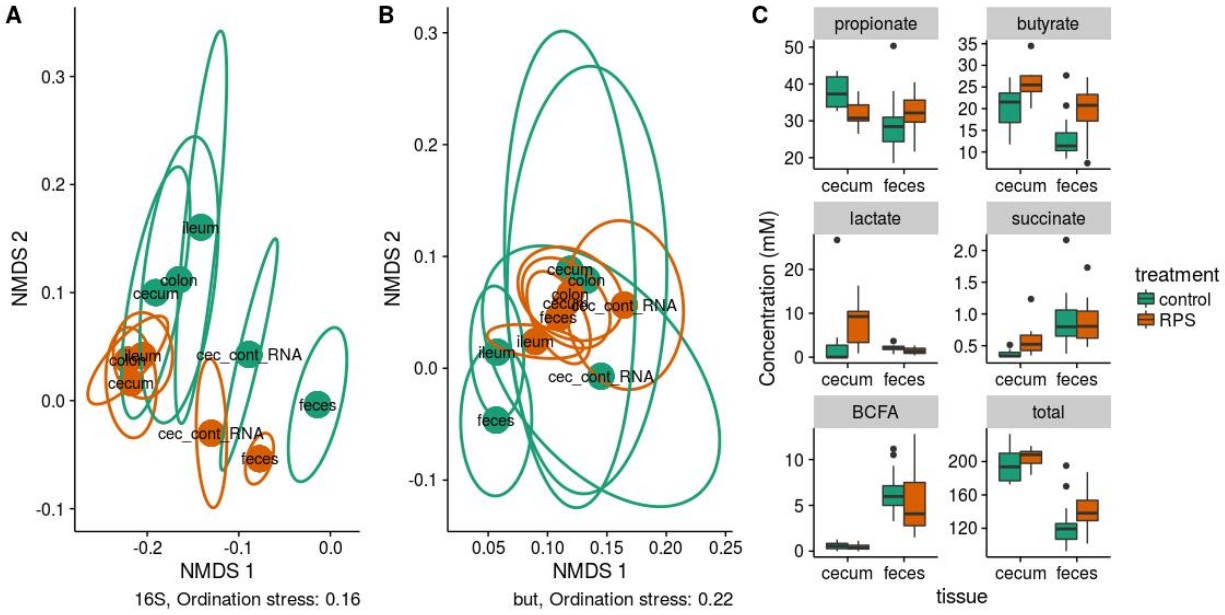

Figure 3: A: NMDS ordinations of 16S rRNA-based Bray-Curtis distances, points indicate group centroids and elipses are standard error. **B:** NMDS ordinations of *but* based Bray-Curtis distances, points indicate group centroids and elipses are standard error. **C:** SCFA concentrations from cecal contents and feces at day 21 post-weaning

Short-chain fatty acid (SCFA) production in the cecum and feces was also evaluated to investigate whether the changes in bacterial membership and genetic potential had an impact on function. Aligning with the changes in microbial communities, the two groups had differing

SCFA profiles (Fig 3C). Pigs fed RPS had higher levels of butyrate in both the cecum and feces ($p=0.053$ & $p=0.048$), lower levels of propionate in the cecum ($p=0.053$), higher levels of the metabolic intermediates lactate and succinate in the cecum ($p=0.09$ & $p=0.01$), and lower levels of lactate in the feces ($p=0.02$). Total SCFA concentrations were not different between the treatment groups in the cecum but we observed significantly increased total fecal SCFAs in the RPS group ($p=0.03$). These results show RPS induced changes in the bacterial communities of many tissues and that these changes affected SCFA production.

Differentially abundant features between the treatment groups at Day 21

At day 21 post-weaning, many bacterial genera were differentially abundant between the two groups (**Figure 4**) in several intestinal tissues (ileum, cecum, colon, and feces). Several genera were consistently associated with each treatment group in all tissues tested. Pigs fed RPS had significantly increased levels of *Terrisporobacter, Sarcina,* and *Clostridium sensu stricto 1* in all tissues. Pigs on the control diet had a significant enrichment of *Mucispirillum* in all tissues as well as sporadic enrichment of many *Proteobacteria* genera such as *Helicobacter, Succinivibrio* and *Campylobacter*. One 16S rRNA gene sequence OTU is responsible for the differential abundance of *Clostriduim sensu stricto 1* between the two groups, which is noteworthy because it suggests that one bacterium, not an entire genus, is sufficiently abundant to be differentially detected with the dietary treatment. OTU00087 was strongly enriched in all tissues and at all timepoints in pigs fed RPS, and was virtually absent from control pigs (**Figure 5**). Sequences from this OTU most closely match *Clostridium chartatabidum*; organisms similar to this bacterial species are shown to tightly bind starch granules(34).

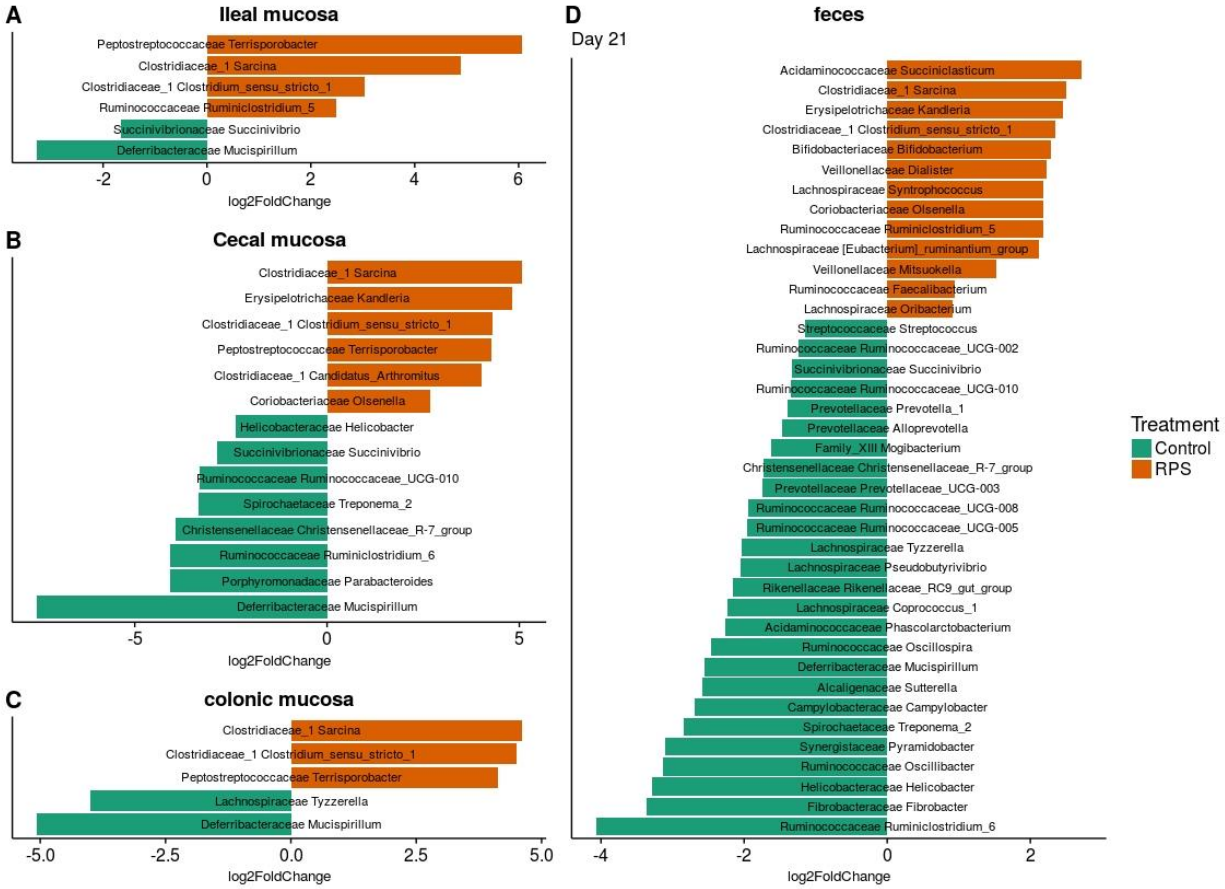

Figure 4: Differentially abundant genera (16S) as determined by DeSeq2.

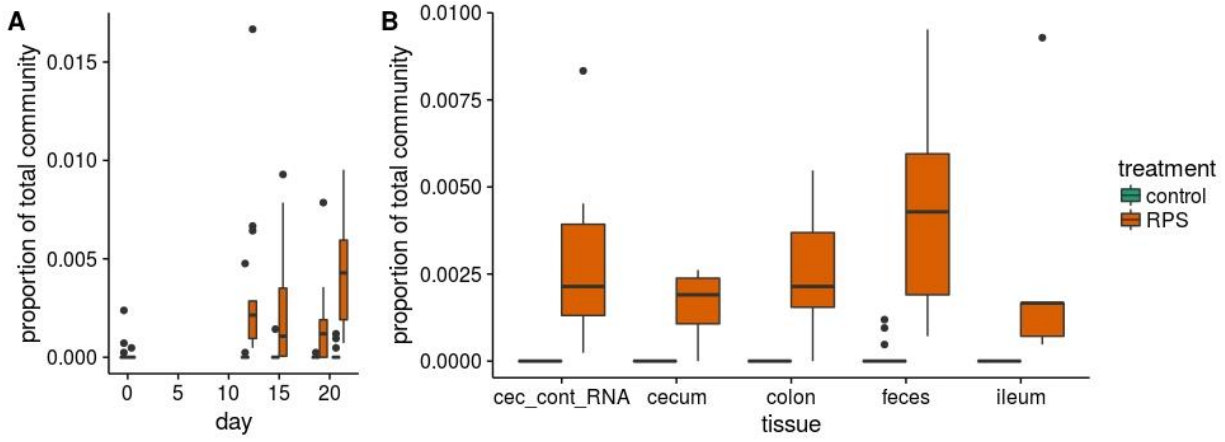

Figure 5: Abundance of OTU87 over time (**A**), and in various tissues (**B**)

Similar to the 16S rRNA gene sequence data, many *but* gene-based OTUs were differentially represented between the two groups (**Figure 6**). RPS intake was consistently associated with a greater abundance of OTUs most closely matching *Anaerostipes hadrus* as well as an OTU that most closely matched an organism only detected in metagenomes from human feces (*but* OTU67). Additionally, several OTUs most closely matching *Eubacterium rectale* were enriched in feces. Control animals had significant enrichments of OTUs most closely matching *Pseudobutyvibrio* and *Helicobacter* in several tissues. These results suggest that certain butyrate-producing bacteria benefited from the dietary inclusion of RPS and were responsible for increased butyrate production in these animals.

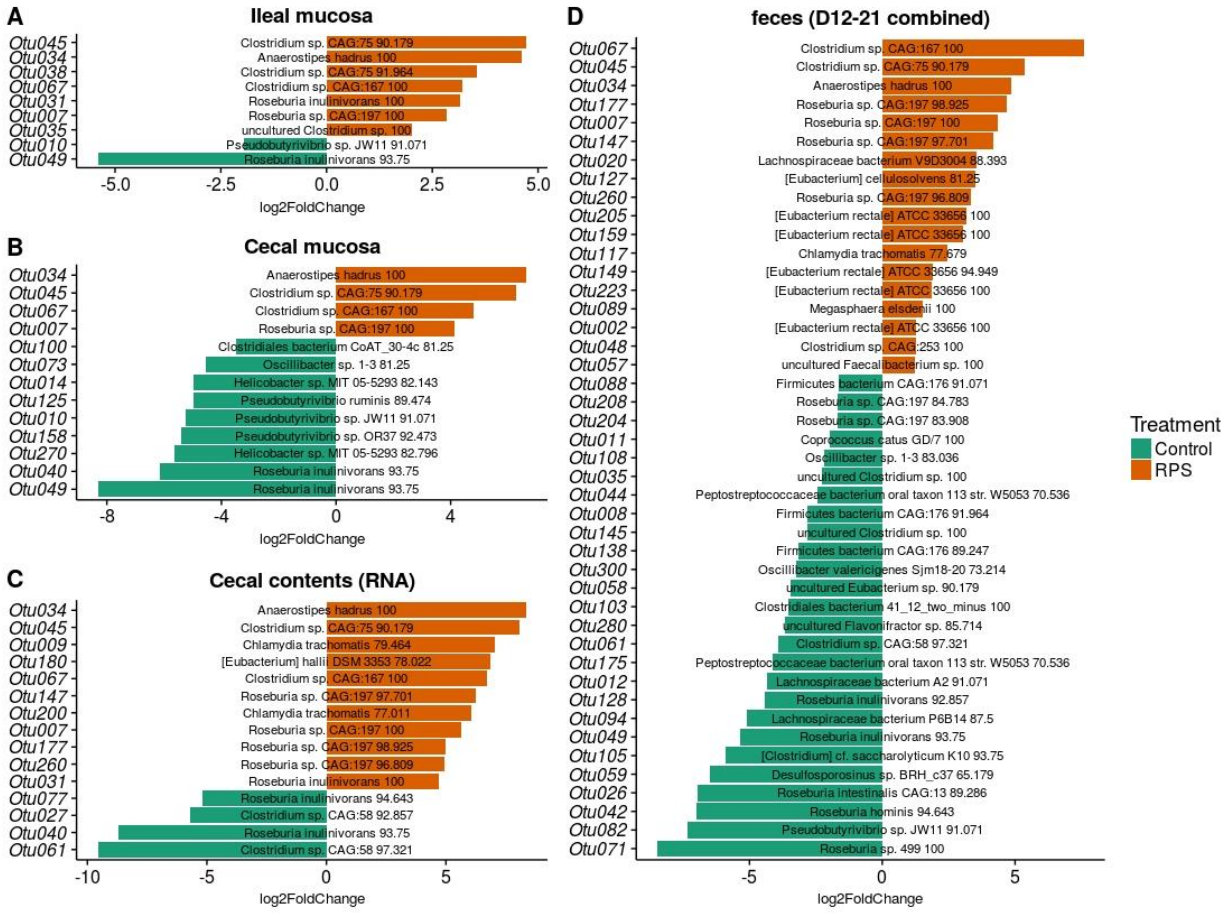

Figure 6: Differentially abundant *but* OTUs as determined by DeSeq2 across various tissues.

Bacterial food webs in the fecal microbiota

To investigate microbial food webs responsible for the increased SCFA production in the RPS animals, we constructed a correlation network using *but* and 16S rRNA gene OTUs (**Figure 7**). Nodes enriched in the RPS treatment group clustered into one primary subnetwork, whereas nodes enriched in the control group clustered into several smaller subnetworks. The network was filtered to only include OTUs that were differentially abundant between the two groups, and so the primary subnetwork enriched in RPS-fed pigs represents bacterial OTUs that are involved in the fermentation of RPS to SCFAs. Bacterial species found to be associated with the RPS-fed swine gut bacterial network are known to be involved in the fermentation of dietary starches, such as *Eubacterium rectale, Mitsuokella* spp., *Prevotella* spp., and *Bifidobacterium* spp. These organisms degrade RPS into small polysaccharides or other metabolic inputs such as lactate, which are then an available carbon source for other bacteria. Lactate-consuming and butyrate-producing bacteria were also enriched in the RPS-fed 16S rRNA gene sequence and *but* gene OTUs network, including *Megasphaera elsdenii*. These results show that dietary RPS supplementation enriches for a bacterial food web that is optimized for degrading dietary fiber and producing SCFAs.

Many butyrate-producing bacteria in gut ecosystems rely on other microbes to convert complex carbohydrate substrates into forms that they can use. The correlation network between *but* gene and 16S rRNA gene sequence OTUs illustrates some of these partnerships. Members of the genus *Prevotella* are known for their ability to breakdown polysaccharides and are highly abundant in swine (35), and we see several 16S rRNA gene sequence OTUs from the *Prevotella 7* group of this genus forming central nodes in the correlation network. This highlights their importance in the early stages of dietary fiber and RPS breakdown, releasing of simple mono-

and oligosaccharides that feed other bacterial species. Additionally, *Bifidobacterium* and *Mitsuokella* OTUs are central nodes in this network as well. These lactate-producing bacteria have been shown to be important in the breakdown of dietary starches and to enhance butyrate production via cross-feeding with butyrate-producers (36). These 16S rRNA-based OTUs correlate with *but*-based OTUs corresponding to well-known butyrate producers such as *Megasphaera elsdenii, Eubacterium rectale, E. cellulosolvens*, and *Faecalibacterium* demonstrating the importance of crossfeeding in butyrate production.

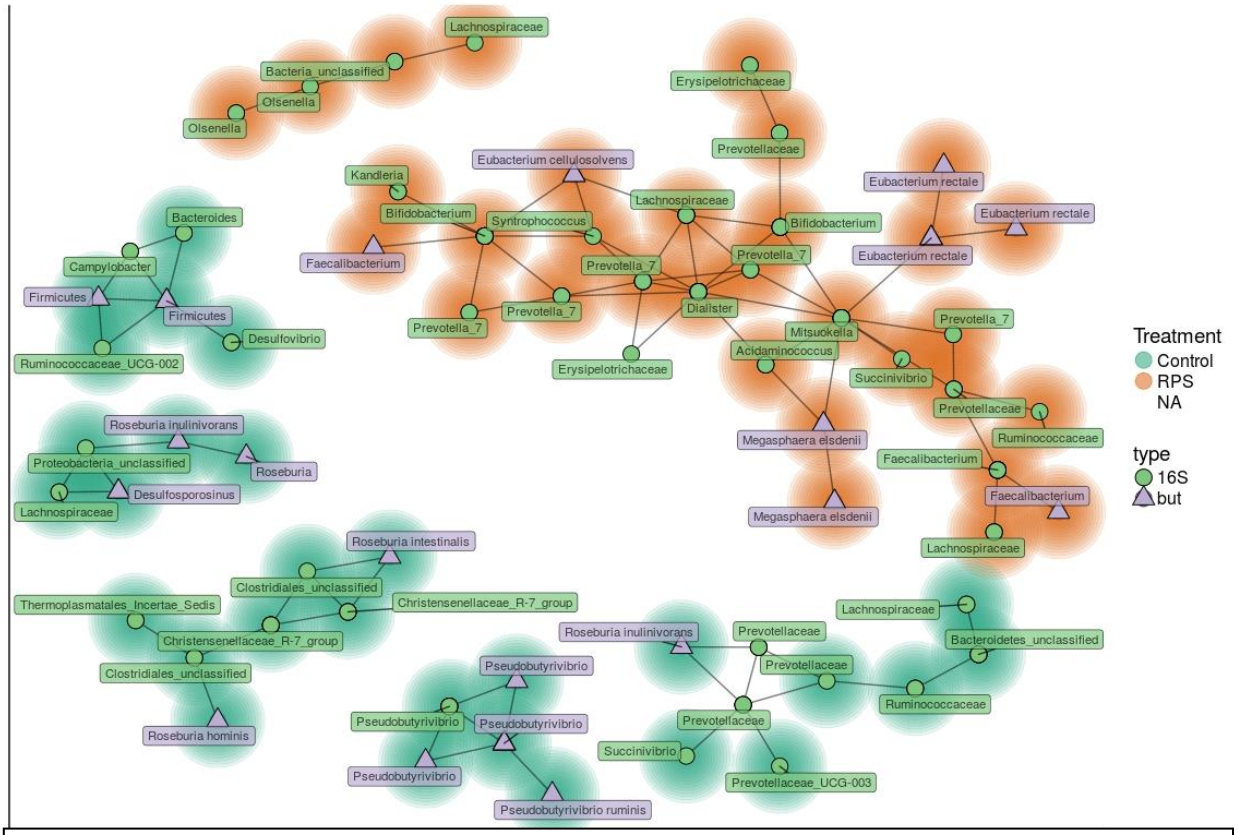

Figure 7: A network depicting correlations between 16S and *but* OTUs in the fecal microbiota. Colors around the nodes indicate which treatment group that particular feature was enriched in as determined by DeSeq2. The network was filtered using the following conditions: 1) Pvalue < 0.05, 2) Spearman correlation coefficient > 0.6, 3) Nodes in a 16S-16S or *but-but* connection must be differentially abundant between treatment groups, 4) At least 1 node in a 16S-*but* connection must be differentially abundant between treatment groups.

Differential host response with dietary RPS

In addition to the many changes in the bacterial communities and metabolites between the two treatment groups, we also observed simultaneous differences in the host immune response. We used a panel of antibodies against CD3, CD4, CD8, CD25, and FoxP3 cellular markers in a flow cytometric assay to identify 16 distinct T-cell populations isolated from the cecal lamina propria, mesenteric lymph nodes, and peripheral blood. The quantity of each cell type was reported as a percent of the total CD3+ cells, which is the general T-cell marker, generating a community data matrix which was used for ecological community analyses. No significant differences were observed in T-cell community structures from the mesenteric lymph nodes or from peripheral blood; however, we observed a significant difference in the T-cell communities residing in the cecal tissues (**Figure 8A)** (PERMANOVA p=0.001, F=12.06). Additionally, the evenness of the cecal T-cell community in control animals was significantly reduced (**Figure 8B,** p = 0.02). Several populations of T-cells were differentially abundant between the two groups (**Figure 8C**), showing that an expansion of CD8+ cell types and a reduction in FoxP3+ cell types in control animals was responsible for the differences in community structure and evenness between the two groups. An additional flow panel was run to determine if significant numbers of gamma delta T cells existed in the CD8a+ population. This

analysis revealed that only ~1% of CD8a+ cells also were positive for the gamma delta TCR in the cecal mucosa (data not shown).

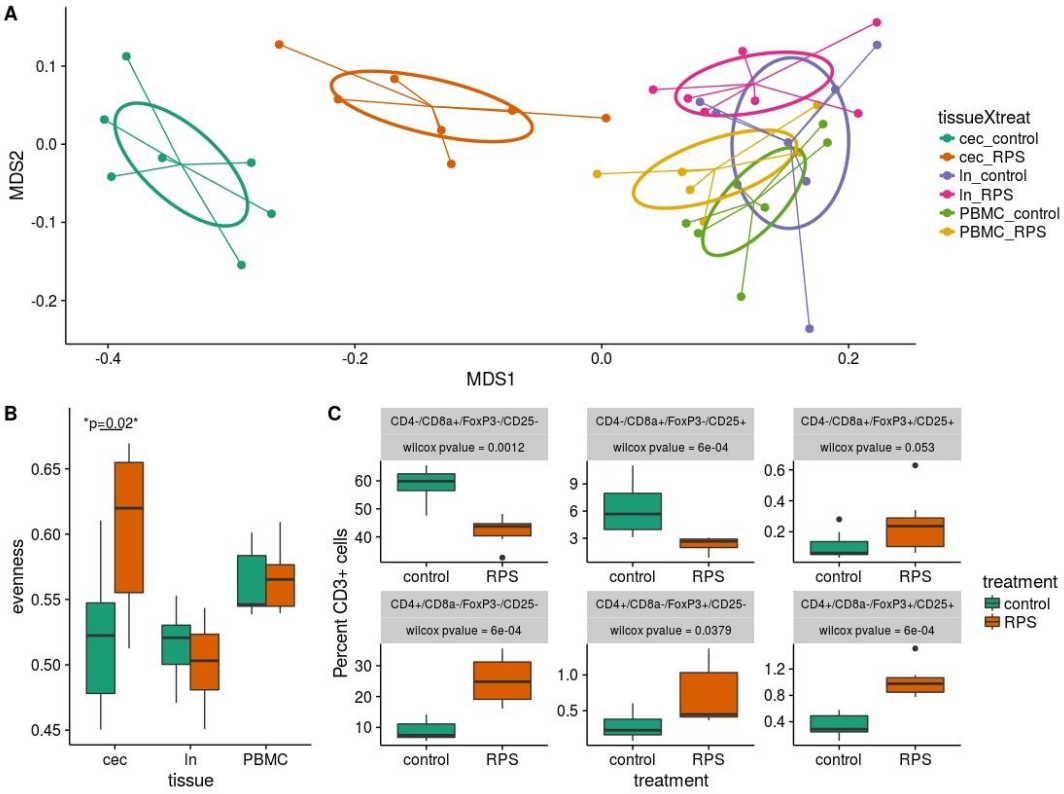

Figure 8: A) A Bray-Curtis based NMDS ordination of T-cell populations in various tissues, **B**) The evenness of the T-cell communities in various tissues, **C**) boxplots of significantly differentially abundant T-cell populations from the cecal T-cell community.

In addition to differences in the cecal T-cell communities, several other differences in the host immune response in the cecum were detected. The expression of host genes involved with mucus production, barrier enhancement, and inflammation was queried. Significantly greater expression of MUC2 and IL-6 was observed, as well as a strong trend towards increased expression of DEF1B, in RPS fed animals compared to the controls (**Figure 9A)**. MUC2 is the primary component of the mucus layer, IL-6 is an important signaling cytokine, and DEF1B is a host-produced antimicrobial peptide. All three of these features are important for the barrier function of the mucosa. In addition, luminal contents were assayed for IgA concentration because IgA is another important host-produced feature that enhances the barrier function of the mucus layer. The results showed a trend towards increased luminal IgA in the RPS fed pigs (**Figure 9B**), increased secretion of IgA and a more robust mucus barrier.

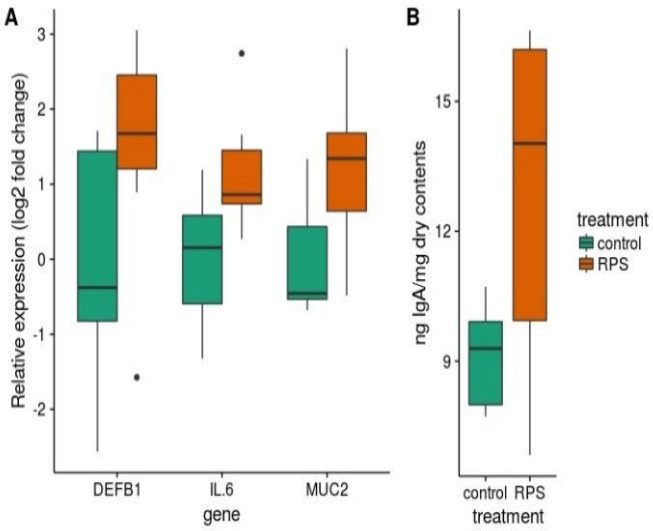

Figure 9: A) mRNA expression of differentially expressed genes from the cecal mucosa. **B**) Amount of IgA in cecal contents

Microbial members and activities correlate with host responses

To investigate correlations between bacterial membership, bacterial function, and host immune responses in the cecum, we constructed a correlation network using the data on cecal immune cell communities (both CD3+ and CD3- cell types were used in this analysis), cecal 16S rRNA gene sequence diversity, cecal SCFA concentrations, cecal host gene expression, and luminal IgA concentrations (**Figure 10**). The results showed one discrete subnetwork associated

with each treatment group. The subnetwork associated with pigs that were fed RPS showed clusters connecting markers of immune tolerance, mucosal barrier function, and anaerobic microbial fermentation. Classical T-regulatory (CD3+/CD4+/CD8-/CD25+/FoxP3+) cells formed a central node in this subnetwork along with several other FoxP3+ cell-types. The SCFAs butyrate, caproate, and valerate correlated with these regulatory immune cells. Bacterial OTUs in this subnetwork belonged to groups known for anaerobic fermentation, and several OTUs correspond to known butyrate-producing groups such as the genus *Megasphaera* and the family *Ruminococcaceae*. In addition, this subnetwork contained nodes associated with promoting the mucosal barrier: MUC2, DEF1B, and IL-6 expression, and high luminal IgA concentrations.

The subnetwork that associated with the control pigs showed markedly different clusters defined by immune activation, cytotoxic immune cells, and bacterial respiration. Not all of the features of this subnetwork were significantly enriched in the control pigs. Only CD3+/CD4-/CD8+/CD25+/FoxP3-, CD3+/CD4-/CD8+/CD25-/FoxP3- cells, propionate, *Succinivibrio*, and *Mucispirillum* met this criteria. The core of this subnetwork was composed of highly interconnected CD25+ immune cell types and a *Campylobacter* OTU, suggesting immune activation and or perhaps an ongoing immune response. Many of the OTUs in this subnetwork belong to the *Proteobacteria* phylum, members of which are known to use respiratory metabolisms (37, 38). Taken together, these results suggest that dietary RPS promotes bacterial food webs that benefit host health by promoting epithelial barrier function and immune tolerance.

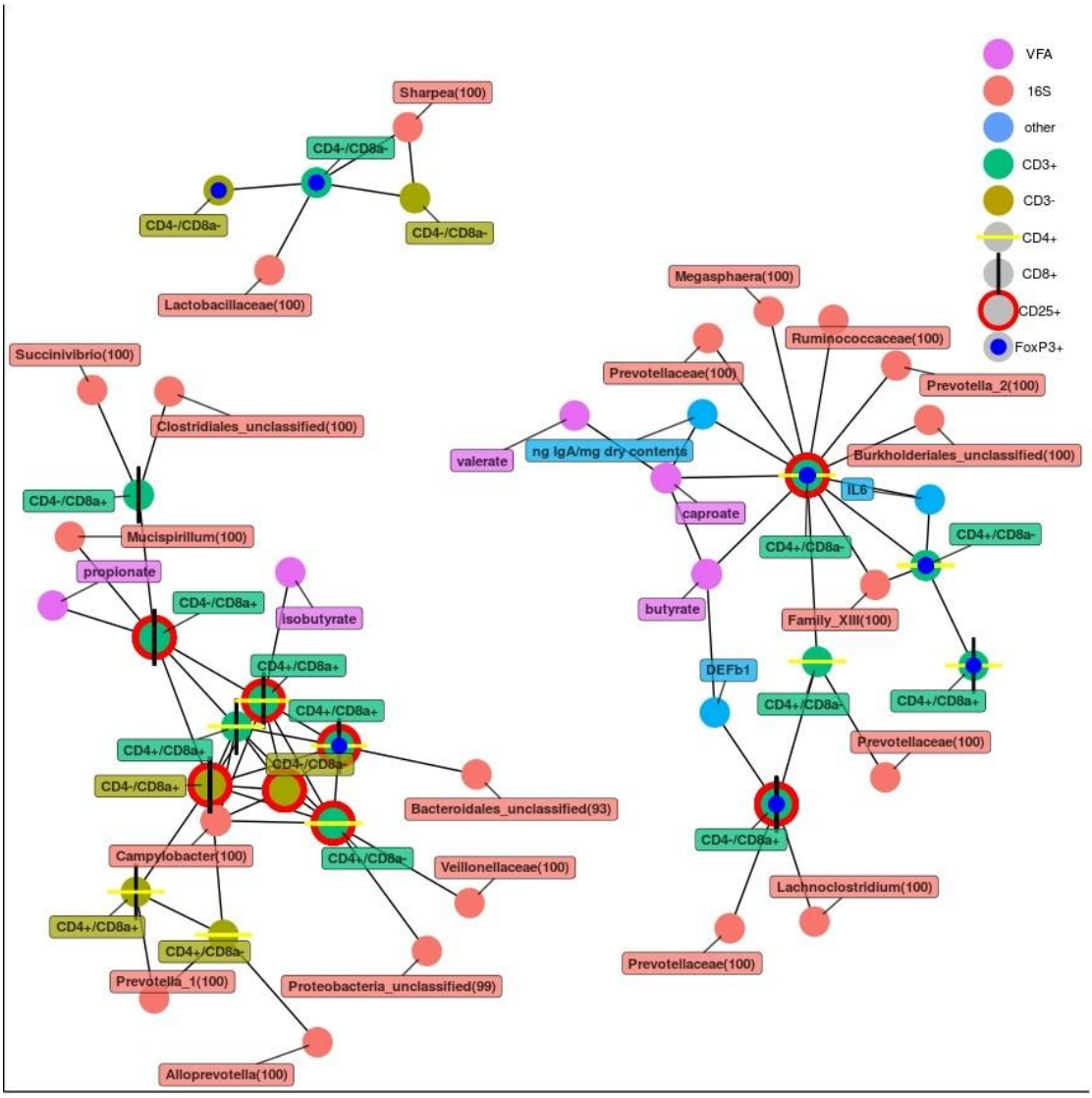

Figure 10: A network depicting correlations among immune cells, 16S rRNA gene sequence diversity, cecal SCFA concentrations, cecal host mRNA expression, and cecal IgA concentrations. The color turquoise is used for the cecal host mRNA expression and IgA concentrations, designated as "other" in the legend. Only positive correlations are shown with a Spearman coefficient >0.6 and a p-value < 0.05.

Discussion

RPS supports microbial-host interactions and enhanced SCFA production

We assessed the effect of dietary RPS on swine gut bacterial diversity, bacterial function, and host immune function. RPS is an easily accessible metabolic input for gut microbes and can encourage increased production of SCFAs, particularly butyrate, which are known to have beneficial effects on the intestinal system. The results showed increased concentrations of cecal butyrate and lactate in the RPS-fed pigs compared to the control animals. In the distal gut lactate is converted to butyrate (36, 39-42), and the lactate observed in the cecum was likely converted to butyrate during colonic passage because fecal lactate concentrations were very low. Additionally, in the RPS-fed pigs we observed an enrichment of butyrate–producing bacteria known to consume lactate. Butyrate is well established as a bacterial metabolite of central importance for intestinal homeostasis and supports many aspects of gut health including reducing the mucosal niche for bacterial respiration (43), increased barrier function (10, 44), water absorption (45), and encouraging a robust yet tolerant immune response to commensal microbes (46-49). This work suggests that RPS intake supports a healthy mucus barrier by stimulating microbial SCFA production, particularly butyrate, which encourages immunological tolerance and a robust mucosal barrier.

In addition to differences mediated by RPS on butyrate-producing microbes, other effects are likely a result of feedback between intestinal bacteria and host tissues. The butyrate produced by gut bacteria is oxidized by host tissues, thereby limiting the amount of oxygen available at the mucosa (43). This establishes a mucosal environment favoring microbial fermentation over respiration, and therefore more SCFA production, and limits inflammation-associated respiratory electron acceptors (21). Consistent with this model, our results showed RPS-

associated microbial food webs are composed of organisms known to use fermentative metabolisms. Well known fermenters such as *Bifidobacteria* spp. and *Faecalibacterium* spp. were enriched in the RPS pigs and are associated with intestinal health in humans (50). Several studies have shown increased populations of these genera in pigs fed resistant starch (7, 51). Other genera enriched in the RPS pigs are less well known for their health benefits. Members of the genus *Clostridium sensu stricto 1* are not generally associated with positive health outcomes; however, our data suggest that members of this genus can be important for resistant starch degradation and intestinal health in the swine gut ecosystem.

The results also showed a positive impact of in-feed RPS on the mucosal barrier in the cecum of weaned piglets. T-cell subtypes and host response profiles associated with intestinal health and mucosal barrier function correlated with microbial butyrate production and anaerobic fermentative microbes in the ceca. Butyrate is known to generate peripheral T-regulatory cells (T-regs) (19, 20, 48), and these T-regs cells were significantly enriched in the RPS group and have been shown to be critical for intestinal homeostasis and gut barrier function(52). Regulatory cells help moderate immune responses to commensal microbes, reducing intestinal inflammation and the mucosal availability of immune-derived electron acceptors, limiting microbial respiration (43, 53). Additionally, the results showed increased concentrations of luminal IgA, and T-regs have been shown to promote the mucosal IgA response, helping the host exert control over its microbial partners (48, 54, 55). CD4-/CD8+/CD25+/FoxP3+ T-cells are less well studied than CD4+ T-regs, but recent work has shown they are an equally important regulatory cell type in humans and mice (56), and our data suggest they are important in swine as well. We see evidence that these host-microbe interactions enhance the mucosal barrier by increasing the expression of MUC2, IL-6, DEFB1 in the cecal mucosa. IL-6 has recently been

shown to be critical for maintaining the mucosal barrier (Kuhn et. al 2017, in review) and has also been shown to be important for strong IgA responses (57). Our data suggest that RPS intake encouraged a protective mucus layer containing more barrier-enhancing molecules such as DEF1B and IgA as well as increased mucosal tolerance and a reduced niche for microbial respiration.

Examination of the microbiota in the control group reveals enriched members that could be detrimental to host health. Bacteria enriched in the control animals have been previously associated with microbial respiration, intestinal inflammation and dysbiosis in humans and mice (58). In particular, members of the genus *Mucispirillum* have been shown to thrive in inflamed, oxygenated mucosal environments. Loy et. al. recently completed a detailed analysis of the genome of *Mucispirillum schaedleri* and showed that this organism utilizes nitrate or free oxygen as terminal electron acceptors for respiration (59). This organism is unable to utilize complex molecules and relies on other microbes to release simple substrates for its consumption (SCFAs, amino acids, monosaccharides etc.), suggesting that it is not a sole causative agent of intestinal dysbiosis but rather an indicator of mucosal inflammation and conditions favoring microbial respiration. Our results showing direct correlations between this microbe and immune cells associated with tissue damage strengthen this connection.

The host response in the control animals is also suggestive of an inflammatory state. The immune cell types that were expanded in the control animals contain cells responsible for cytotoxic activity, although we did not detect any well-known intracellular pathogens in our 16S rRNA gene sequence analysis of the cecal mucosa. However, we did detect consistent enrichment of *Helicobacter* in control animal tissues, and bacteria from this genus have been shown to be facultative intracellular pathogens (60) , with *H. canadensis* shown to cause diarrhea

in humans (61), and has been previously detected in swine (62). Sequences detected in our pigs most closely match *H. equorum (*100% V4 identity) and *H. canadensis* (99.8% V4 identity). A recent study suggests that non-*H. pylori Helicobacter* species may be a cause of irritable bowel disease (IBD) in humans (63). It is likely that there is not one causative agent for the expansion of cytotoxic T-cell subtypes, but their presence suggests a compromised mucosal barrier and the invasion of the piglet mucosa by the commensal microbiota causing tissue damage and the CD8+ cell expansion.

The RPS diet did not completely protect pigs from experiencing immune activation towards intestinal commensal bacteria. Many nodes belonging to the immune activation subnetwork were equally represented in both groups. For example, Double positive CD25+ T-cells are prominent members of this immune activation subnetwork and were not differentially abundant between the two groups. These cells have been shown to be common in swine and are highly activated, primed lymphocytes (64, 65). Similarly, two proteobacterial species were found to correlate with CD3+CD4+CD8a-CD25+FoxP3- cells, a *Campylobacter* OTU, and an unclassified *Proteobacteria* OTU. This finding supports the idea that many P*roteobacteria* species exploit or thrive under conditions of inflammation in swine. As these results are only correlations it is not clear if these OTUs are the cause of the inflammation or are merely thriving in conditions brought on by inflammation, or some combination of both. This suggests that not all individuals react in the same way to dietary prebiotics such as RPS, a concept that has been demonstrated in humans and mice (66). To optimize the health benefits of RPS it may be necessary to co-administer some of the RPS-degrading bacteria identified in this study to aid in the establishment of beneficial bacterial food webs and interactions with the host.

Weaning-associated changes in the microbiota and potential challenges

In addition to the dietary modulation by RPS, the experimental design also yielded information regarding microbiota changes after weaning. The transition from a milk-based diet to a solid corn-based diet induced a major shift in the swine gut microbiota, as has been reported in other studies (67, 68). This shifting microbiota leaves the piglet susceptible to opportunistic infections. Previous work has shown that weaning is associated with transient intestinal inflammation (69), and health problems associated with weaning are well documented (1). As the microbiota matures, the intestinal ecosystem becomes more resilient to perturbations and develops inherent resistance to colonization by new microbes or pathogens (70). One way to support the piglet's intestinal microbiota to be resistant to perturbations and disease is to establish a healthy bacterial community. The data presented in this paper show that dietary RPS promotes bacterial fermentation and supports the host mucosal functions, suggesting that dietary RPS could support gut microbiota functions associated with health as the piglet and its microbiota mature.

As the bacterial populations change after weaning, so do the enzymatic capabilities of the fecal microbiota. The results show that the glycan utilization of the intestinal microbiota changes in parallel with the change in bacterial community composition. Rates for the breakdown of plant-derived sugar linkages increased through the 21 days post-weaning, whereas enzymatic activities to mucin-derived substrates showed the opposite trend. High activity towards fucose-containing polymers, as shown in our results, is associated with bacterial growth on mucins (71), and the cleavage of fucose by commensal bacteria has been shown to benefit the growth of some pathogens (72). These data show that the enzymatic potential of the fecal microbiota is changing throughout the first 3 weeks post-weaning. Furthermore, these data show

that early in weaning, the intestinal microbiota degrades the host mucus barrier as an energy source as it adjusts to the solid corn-based diet, suggesting one potential cause of common weaning-associated intestinal distress in piglets.

Conclusions

Our study reveals the specific microbial interactions involved in the microbial breakdown of resistant starch and identifies specific bacterial groups which could be co-administered with RPS to enhance its effects. Additionally, this work reveals both beneficial and potentially negative interactions between the commensal microbiota and mucosal immune system that can inform future strategies to mitigate weaning-associated complications. Together, these data provide valuable insights into the host-microbe interactions in the intestinal mucosa of swine and will help inform management practices with the potential to benefit both animal health and food safety.

Supplemental Material

Table S1: Composition of diets. All diets were formulated at Iowa State University.

	Non-Amended Phase I Diet	Amended Phase 1 Diet	Non-Amended Phase 2 Diet	Amended Phase 2 Diet
Corn, yellow dent	49.00%	44.00%	61.06%	56.06%
Soybean meal (46.5% CP*)	28.20%	28.20%	27.00%	27.00%
Casein	2.70%	2.70%		-
Lactose	10.00%	10.00%	2.50%	2.50%
Fish meal (menhaden)	4.50%	4.50%	4.66%	4.66%
Soybean oil	2.00%	2.00%	1.65%	1.65%
L-lysine HCl	0.35%	0.35%	0.38%	0.38%
DL-methionine	0.15%	0.15%	0.12%	0.12%
L-threonine	0.13%	0.13%	0.12%	0.12%
L-tryptophan	0.02%	0.02%	0.01%	0.01%
L-valine	0.11%	0.11%	-	-
Monocalcium phosphate (21%)	1.15%	1.15%	0.96%	0.96%
Limestone	0.80%	0.80%	0.74%	0.74%
Salt	0.50%	0.50%	0.40%	0.40%
NSNG Nursery Vitamin Premix	0.25%	0.25%	0.25%	0.25%
NSNG Trace Mineral Mix[a]	0.15%	0.15%	0.15%	0.15%
MSP[RS][b] Raw Potato Starch	-	5.00%	-	5.00%

[a]: National Swine Nutrition Guide

[b]: Manitoba Starch Products Resistant Starch

*: Crude Protein

Table S2: TaqMan® Gene Expression Targets for qRT-PCR.

Symbol	Gene Name	Accession	Assay ID	Function
DEFB1	defensin β1	NM_213838	Ss03381769_u1	antimicrobial peptide
FFAR2	free fatty acid receptor 2	NM_001278758	Ss03374174_s1	SCFA receptor
IL-10	interleukin-10	NM_214041	Ss03382372_u1	cytokine signaling
IL-17	interleukin-17A	NM_001005729	Ss03391803_m1	cytokine signaling
IL-22	interleukin-22	AY937228	Ss03373919_m1	cytokine signaling
IL-1β	interleukin-1β	NM_214055	Ss03393804_m1	cytokine signaling
IL-6	interleukin-6	NM_214399	Ss03384604_u1	cytokine signaling
MUC2	small intestinal mucin	EU143549	Ss03377386_u1	mucus layer defense
PR39	PR-39	NM_214450	Ss03385004_u1	antimicrobial peptide
TGFβ	transforming growth factor β	NM_214015	Ss03382325_u1	cytokine signaling
ACTβ	β -actin	AK237086	Ss03376081_u1	reference gene

All primers were labeled with FAM dye and specific for s*us scrofa* (Thermo Fisher Scientific).

Figure S1: Representative flow plot used for determining frequency of T cell populations in the cecum. 1A) Live cells gating (negative for stain), 1B) $CD3^+$ gating 1C) CD4 and Cd8α gating of live $CD3^+$ cells and 1D) representative gating for CD25 and FoxP3 staining of indicated T cell populations - the blue dots are representative of $CD4^+$/ $CD8\alpha^-$ population and the red dots are representative of $CD4^+$/ $CD8\alpha^+$ population.

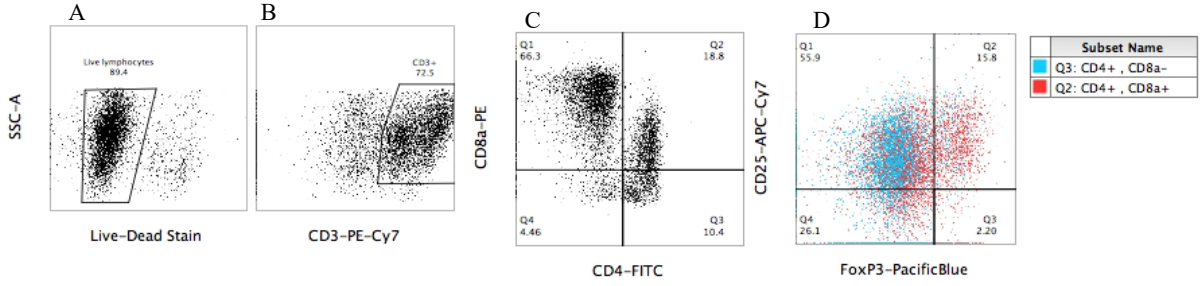

Table S3: PERMANOVA results showing treatment group differences in community structure across time and tissues. Both 16S and *but* based communities. Calculated with vegan's Adonis function.

Comparison	F.Model	R2	p.value	gene	day	tissue
feces_0_control vs feces_0_RPS	0.597851	0.022477	0.9073	16S	0	feces
feces_12_control vs feces_12_RPS	0.974797	0.037529	0.4307	16S	12	feces
feces_15_control vs feces_15_RPS	1.94773	0.072278	0.0159	16S	15	feces
feces_19_control vs feces_19_RPS	2.607228	0.105954	0.0017	16S	19	feces
feces_21_control vs feces_21_RPS	5.257901	0.179709	1.00E-04	16S	21	feces
cec_cont_RNA_21_control vs cec_cont_RNA_21_RPS	1.860549	0.134233	0.0566	16S	21	cec_cont_RNA
cecum_21_control vs cecum_21_RPS	2.64312	0.180503	0.0085	16S	21	cecum
colon_21_control vs colon_21_RPS	1.441706	0.107256	0.1439	16S	21	colon
ileum_21_control vs ileum_21_RPS	2.67778	0.308579	0.0367	16S	21	ileum
feces_0_control vs feces_0_RPS	0.794	0.029633	0.5793	*but*	0	feces
feces_12_control vs feces_12_RPS	0.746653	0.027916	0.7542	*but*	12	feces
feces_15_control vs feces_15_RPS	1.522014	0.057387	0.0925	*but*	15	feces
feces_19_control vs feces_19_RPS	3.582779	0.151924	1.00E-04	*but*	19	feces
feces_21_control vs feces_21_RPS	3.880003	0.129853	3.00E-04	*but*	21	feces
cec_cont_RNA_21_control vs cec_cont_RNA_21_RPS	1.694801	0.133504	0.054	*but*	21	cec_cont_RNA
cecum_21_control vs cecum_21_RPS	1.859664	0.134178	0.0483	*but*	21	cecum
colon_21_control vs colon_21_RPS	1.36554	0.102169	0.2063	*but*	21	colon
ileum_21_control vs ileum_21_RPS	2.333303	0.162789	0.0112	*but*	21	ileum

Table S4: Community dispersions across time and tissues within and between groups for both 16S and *but* communities. Calculated with a permutation test of vegan's betadisper function.

Comparison	16S p value	but p value
cec_cont_RNA_21_control vs cec_cont_RNA_21_RPS	0.886	0.028
cecum_21_control vs cecum_21_RPS	0.007	0.012
colon_21_control vs colon_21_RPS	0.129	0.078
feces_0_control vs feces_0_RPS	0.942	0.714
feces_0_control vs feces_12_control	0.299	0.758
feces_0_control vs feces_15_control	0.001	0.744
feces_0_control vs feces_19_control	0.003	0.064
feces_0_control vs feces_21_control	0.001	0.223
feces_0_RPS vs feces_12_RPS	0.042	0.962
feces_0_RPS vs feces_15_RPS	0.001	0.25
feces_0_RPS vs feces_19_RPS	0.009	0.353
feces_0_RPS vs feces_21_RPS	0.001	0.013
feces_12_control vs feces_12_RPS	0.351	0.873
feces_12_control vs feces_15_control	0.015	0.464
feces_12_control vs feces_19_control	0.081	0.02
feces_12_control vs feces_21_control	0.183	0.065
feces_12_RPS vs feces_15_RPS	0.189	0.139
feces_12_RPS vs feces_19_RPS	0.429	0.181
feces_12_RPS vs feces_21_RPS	0.001	0.004
feces_15_control vs feces_15_RPS	0.272	0.686
feces_15_control vs feces_19_control	0.341	0.108
feces_15_control vs feces_21_control	0.175	0.363
feces_15_RPS vs feces_19_RPS	0.748	0.775
feces_15_RPS vs feces_21_RPS	0.001	0.075
feces_19_control vs feces_19_RPS	0.839	0.055
feces_19_control vs feces_21_control	0.645	0.283
feces_19_RPS vs feces_21_RPS	0.001	0.041
feces_21_control vs feces_21_RPS	0.003	0.175
ileum_21_control vs ileum_21_RPS	0.799	0.569

Citations

1. Heo JM, Opapeju FO, Pluske JR, Kim JC, Hampson DJ, Nyachoti CM. 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J Anim Physiol Anim Nutr (Berl) 97:207-37.
2. USDA. 2015. "Part II: Reference of Swine Health and Health Management in the United States, 2012". USDA–APHIS–VS–CEAH–NAHM, USDA, Fort Collins, CO.
3. Louis P, Flint HJ, Michel C. 2016. How to Manipulate the Microbiota: Prebiotics. Adv Exp Med Biol 902:119-42.
4. Haenen D, Zhang J, Souza da Silva C, Bosch G, van der Meer IM, van Arkel J, van den Borne JJ, Perez Gutierrez O, Smidt H, Kemp B, Muller M, Hooiveld GJ. 2013. A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine. J Nutr 143:274-83.
5. Giuberti G, Gallo A, Moschini M, Masoero F. 2015. New insight into the role of resistant starch in pig nutrition. Animal Feed Science and Technology 201:1-13.
6. Sun Y, Zhou L, Fang L, Su Y, Zhu W. 2015. Responses in colonic microbial community and gene expression of pigs to a long-term high resistant starch diet. Front Microbiol 6:877.
7. Umu OC, Frank JA, Fangel JU, Oostindjer M, da Silva CS, Bolhuis EJ, Bosch G, Willats WG, Pope PB, Diep DB. 2015. Resistant starch diet induces change in the swine microbiome and a predominance of beneficial bacterial populations. Microbiome 3:16.
8. Sun Y, Su Y, Zhu W. 2016. Microbiome-Metabolome Responses in the Cecum and Colon of Pig to a High Resistant Starch Diet. Front Microbiol 7:779.
9. Desai MS, Seekatz AM, Koropatkin NM, Kamada N, Hickey CA, Wolter M, Pudlo NA, Kitamoto S, Terrapon N, Muller A, Young VB, Henrissat B, Wilmes P, Stappenbeck TS, Núñez G, Martens EC. 2016. A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. Cell 167:1339-1353.e21.
10. Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, Wilson KE, Glover LE, Kominsky DJ, Magnuson A, Weir TL, Ehrentraut SF, Pickel C, Kuhn KA, Lanis JM, Nguyen V, Taylor CT, Colgan SP. 2015. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. Cell Host Microbe 17:662-71.
11. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res 54:2325-40.
12. Kim M, Qie Y, Park J, Kim CH. 2016. Gut Microbial Metabolites Fuel Host Antibody Responses. Cell Host Microbe 20:202-14.
13. Zeng X, Sunkara LT, Jiang W, Bible M, Carter S, Ma X, Qiao S, Zhang G. 2013. Induction of porcine host defense peptide gene expression by short-chain fatty acids and their analogs. PLoS One 8:e72922.
14. Xiong H, Guo B, Gan Z, Song D, Lu Z, Yi H, Wu Y, Wang Y, Du H. 2016. Butyrate upregulates endogenous host defense peptides to enhance disease resistance in piglets via histone deacetylase inhibition. Sci Rep 6:27070.
15. Schilderink R, Verseijden C, Seppen J, Muncan V, van den Brink GR, Lambers TT, van Tol EA, de Jonge WJ. 2016. The SCFA butyrate stimulates the epithelial production of

retinoic acid via inhibition of epithelial HDAC. Am J Physiol Gastrointest Liver Physiol 310:G1138-46.
16. Zimmerman MA, Singh N, Martin PM, Thangaraju M, Ganapathy V, Waller JL, Shi H, Robertson KD, Munn DH, Liu K. 2012. Butyrate suppresses colonic inflammation through HDAC1-dependent Fas upregulation and Fas-mediated apoptosis of T cells. Am J Physiol Gastrointest Liver Physiol 302:G1405-15.
17. Fontenelle B, Gilbert KM. 2012. n-Butyrate anergized effector CD4+ T cells independent of regulatory T cell generation or activity. Scand J Immunol 76:457-63.
18. Harrison OJ, Powrie FM. 2013. Regulatory T cells and immune tolerance in the intestine. Cold Spring Harb Perspect Biol 5.
19. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, Ohno H. 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature 504:446-50.
20. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 504:451-5.
21. Spees AM, Lopez CA, Kingsbury DD, Winter SE, Baumler AJ. 2013. Colonization resistance: battle of the bugs or me´nage a`trois with the host? PLOS Pathogens 9:1-4.
22. Rivera-Chavez F, Winter SE, Lopez CA, Xavier MN, Winter MG, Nuccio SP, Russell JM, Laughlin RC, Lawhon SD, Sterzenbach T, Bevins CL, Tsolis RM, Harshey R, Adams LG, Baumler AJ. 2013. *Salmonella* uses energy taxis to benefit from intestinal inflammation. PLoS Pathog 9:e1003267.
23. Heo JM, Agyekum AK, Yin YL, Rideout TC, Nyachoti CM. 2014. Feeding a diet containing resistant potato starch influences gastrointestinal tract traits and growth performance of weaned pigs. Journal Of Animal Science 92:3906-3913.
24. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol 79:5112-20.
25. Trachsel J, Bayles DO, Looft T, Levine UY, Allen HK. 2016. Function and Phylogeny of Bacterial Butyryl Coenzyme A:Acetate Transferases and Their Diversity in the Proximal Colon of Swine. Appl Environ Microbiol 82:6788-6798.
26. Salanitro JP, Muirhead PA. 1975. Quantitative Method for the Gas Chromatographic Analysis of Short-Chain Monocarboxylic and Dicarboxylic Acids in Fermentation Media. Applied Microbiology 29:374-381.
27. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537-41.
28. Fish JA, Chai B, Wang Q, Sun Y, Brown CT, Tiedje JM, Cole JR. 2013. FunGene: the functional gene pipeline and repository. Front Microbiol 4:291.
29. Jari Oksanen, F. Guillaume Blanchet, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R. Minchin, R. B. O'Hara, Gavin L. Simpson, Peter Solymos, M.

Henry H. Stevens, Eduard Szoecs, Helene Wagner. 2017. vegan: Community Ecology Package, vR package version 2.4-2. https://CRAN.R-project.org/package=vegan.

30. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15:550.
31. Emma schwager, Craig B, Weingart G. 2014. ccrepe: ccrepe_and_nc.score, vR package version 1.12.1.
32. Jr. FEH, Dupont C. 2016. Hmisc: Harrell Miscellaneous, vR package version 4.0-2. https://CRAN.R-project.org/package=Hmisc.
33. Tyner S, Briatte F, Hofmann H. 2017. Network Visualization with ggplot. The R Journal.
34. Sharp R, Macfarlane GT. 2000. Chemostat Enrichments of Human Feces with Resistant Starch Are Selective for Adherent Butyrate-Producing Clostridia at High Dilution Rates. Applied and Environmental Microbiology 66:4212-4221.
35. Accetto T, Avgustin G. 2015. Polysaccharide utilization locus and CAZYme genome repertoires reveal diverse ecological adaptation of Prevotella species. Syst Appl Microbiol 38:453-61.
36. Scott KP, Martin JC, Duncan SH, Flint HJ. 2014. Prebiotic stimulation of human colonic butyrate-producing bacteria and bifidobacteria, in vitro. FEMS Microbiol Ecol 87:30-40.
37. Kern M, Winkler C, Simon J. 2011. Respiratory nitrogen metabolism and nitrosative stress defence in -proteobacteria: the role of NssR-type transcription regulators. Biochem Soc Trans 39:299-302.
38. Ravcheev DA, Gerasimova AV, Mironov AA, Gelfand MS. 2007. Comparative genomic analysis of regulation of anaerobic respiration in ten genomes from three families of gamma-proteobacteria (Enterobacteriaceae, Pasteurellaceae, Vibrionaceae). BMC Genomics 8:54.
39. Duncan SH, Louis P, Flint HJ. 2004. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl Environ Microbiol 70:5810-7.
40. Belenguer A, Duncan SH, Calder AG, Holtrop G, Louis P, Lobley GE, Flint HJ. 2006. Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. Appl Environ Microbiol 72:3593-9.
41. Falony G, Vlachou A, Verbrugghe K, De Vuyst L. 2006. Cross-feeding between Bifidobacterium longum BB536 and acetate-converting, butyrate-producing colon bacteria during growth on oligofructose. Appl Environ Microbiol 72:7835-41.
42. Bourriaud C, Robins RJ, Martin L, Kozlowski F, Tenailleau E, Cherbut C, Michel C. 2005. Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident. Journal of Applied Microbiology 99:201-212.
43. Rivera-Chavez F, Zhang LF, Faber F, Lopez CA, Byndloss MX, Olsan EE, Xu G, Velazquez EM, Lebrilla CB, Winter SE, Baumler AJ. 2016. Depletion of butyrate-producing *Clostridia* from the gut microbiota drives an aerobic luminal expansion of *Salmonella*. Cell Host Microbe 19:443-54.
44. Kim JC, Hansen CF, Mullan BP, Pluske JR. 2012. Nutrition and pathology of weaner pigs: Nutritional strategies to support barrier function in the gastrointestinal tract. Animal Feed Science and Technology 173:3-16.
45. SANDLE GI. 1998. Salt and water absorption in the human colon: a modern appraisal. Gut 43:294-299.

46. Hooper LV, Littman DR, Macpherson AJ. 2012. Interactions between the microbiota and the immune system. Science 336:1268-1273.
47. Chang PV, Hao L, Offermanns S, Medzhitov R. 2014. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proc Natl Acad Sci U S A 111:2247-52.
48. Zeng H, Chi H. 2015. Metabolic control of regulatory T cell development and function. Trends Immunol 36:3-12.
49. Schilderink R, Verseijden C, Seppen J, Muncan V, Brink GRvd, Lambers TT, Tol EAv, Jonge WJd. 2016. The SCFA butyrate stimulates epithelial production of retinoic acid via inhibition of epithelial HDAC. Am J Physiol Gastrointest Liver Physiol 310:G1138-G1146.
50. Riviere A, Selak M, Lantin D, Leroy F, De Vuyst L. 2016. Bifidobacteria and Butyrate-Producing Colon Bacteria: Importance and Strategies for Their Stimulation in the Human Gut. Front Microbiol 7:979.
51. Fouhse JM, Ganzle MG, Regmi PR, van Kempen TA, Zijlstra RT. 2015. High Amylose Starch with Low In Vitro Digestibility Stimulates Hindgut Fermentation and Has a Bifidogenic Effect in Weaned Pigs. J Nutr doi:10.3945/jn.115.214353.
52. Tanoue T, Atarashi K, Honda K. 2016. Development and maintenance of intestinal regulatory T cells. Nat Rev Immunol 16:295-309.
53. Kelly Caleb J, Colgan Sean P. Breathless in the Gut: Implications of Luminal O₂ for Microbial Pathogenicity. Cell Host & Microbe 19:427-428.
54. Suzuki K, Ha SA, Tsuji M, Fagarasan S. 2007. Intestinal IgA synthesis: a primitive form of adaptive immunity that regulates microbial communities in the gut. Semin Immunol 19:127-35.
55. Kubinak JL, Petersen C, Stephens WZ, Soto R, Bake E, O'Connell RM, Round JL. 2015. MyD88 signaling in T cells directs IgA-mediated control of the microbiota to promote health. Cell Host Microbe 17:153-63.
56. Churlaud G, Pitoiset F, Jebbawi F, Lorenzon R, Bellier B, Rosenzwajg M, Klatzmann D. 2015. Human and Mouse CD8+CD25+FOXP3+ Regulatory T Cells at Steady State and during Interleukin-2 Therapy. Frontiers in Immunology 6.
57. Ramsay AJ, Husband AJ, Ramshaw IA, Bao S, Matthaei KI, Koehler G, Kopf M. 1994. The role of interleukin-6 in mucosal IgA antibody responses in vivo. Science 264:561-3.
58. Rooks MG, Veiga P, Wardwell-Scott LH, Tickle T, Segata N, Michaud M, Gallini CA, Beal C, van Hylckama-Vlieg JE, Ballal SA, Morgan XC, Glickman JN, Gevers D, Huttenhower C, Garrett WS. 2014. Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission. ISME J 8:1403-17.
59. Loy A, Pfann C, Steinberger M, Hanson B, Herp S, Brugiroux S, Gomes Neto JC, Boekschoten MV, Schwab C, Urich T, Ramer-Tait AE, Rattei T, Stecher B, Berry D. 2017. Lifestyle and Horizontal Gene Transfer-Mediated Evolution of Mucispirillum schaedleri, a Core Member of the Murine Gut Microbiota. mSystems 2.
60. Dubois A, Borén T. 2007. Helicobacter pylori is invasive and it may be a facultative intracellular organism. Cellular microbiology 9:1108-1116.
61. Fox JG, Chien CC, Dewhirst FE, Paster BJ, Shen Z, Melito PL, Woodward DL, Rodgers FG. 2000. Helicobacter canadensis sp. nov. isolated from humans with diarrhea as an example of an emerging pathogen. J Clin Microbiol 38:2546-9.

62. Inglis GD, McConville M, de Jong A. 2006. Atypical Helicobacter canadensis Strains Associated with Swine. Applied and Environmental Microbiology 72:4464-4471.
63. Yu Q, Zhang S, Li L, Xiong L, Chao K, Zhong B, Li Y, Wang H, Chen M. 2015. Enterohepatic Helicobacter Species as a Potential Causative Factor in Inflammatory Bowel Disease: A Meta-Analysis. Medicine (Baltimore) 94:e1773.
64. Pahar B, Lackner AA, Veazey RS. 2006. Intestinal double-positive CD4+CD8+ T cells are highly activated memory cells with an increased capacity to produce cytokines. Eur J Immunol 36:583-92.
65. Zuckermann FA. 1999. Extrathymic CD4/CD8 double positive T cells. Vet Immunol Immunopathol 72:55-66.
66. Venkataraman A, Sieber JR, Schmidt AW, Waldron C, Theis KR, Schmidt TM. 2016. Variable responses of human microbiomes to dietary supplementation with resistant starch. Microbiome 4:33.
67. Mach N, Berri M, Estellé J, Levenez F, Lemonnier G, Denis C, Leplat J-J, Chevaleyre C, Billon Y, Doré J, Rogel-Gaillard C, Lepage P. 2015. Early life establishment of the swine gut microbiome 1 and impact on host phenotypes. Environmental Microbiology doi:10.1111/17582229.12285.
68. Pajarillo E, Chae JP, MP B, HB K, Kang DK. 2014. Assessment of fecal bacterial diversity among healthy piglets during the weaning transition. J Gen Appl Microbiol 60.
69. Pie S, Lalles JP, Blazy F, Laffitte J, Seve B, Oswald IP. 2004. Weaning Is Associated with an Upregulation of Expression of Inflammatory Cytokines in the Intestine of Piglets. Journal of Nutrition 134:641-647.
70. Lawley TD, Walker AW. 2013. Intestinal colonization resistance. Immunology 138:1-11.
71. Robertson BR, O'Rourke JL, Neilan BA, Vandamme P, On SL, Fox JG, Lee A. 2005. Mucispirillum schaedleri gen. nov., sp. nov., a spiral-shaped bacterium colonizing the mucus layer of the gastrointestinal tract of laboratory rodents. Int J Syst Evol Microbiol 55:1199-204.
72. Ng KM, Ferreyra JA, Higginbottom SK, Lynch JB, Kashyap PC, Gopinath S, Naidu N, Choudhury B, Weimer BC, Monack DM, Sonnenburg JL. 2013. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. Nature 502:96-99.

CHAPTER 5: PROGRESS TOWARDS RESEARCH GOALS AND DIRECTIONS FOR FUTURE WORK

Summary

Development of non-antibiotic-based approaches and strategies to support the health of commercially-important livestock species is essential. Increasingly, the spread of antimicrobial resistance genes is becoming both a food safety and animal health concern, and efforts to mitigate this risk are important. Consequently, investigating ways to improve swine health without the use of antibiotics is a central research topic. Gut health is critical to the overall wellbeing of an animal, so the intestinal microbiome is a potential intervention point for modulating health. However, mechanistic knowledge of the complexities of the host-microbial ecosystem is lacking, and this impedes the rational design of strategies to modulate the microbiota. In this final chapter, specific advancements contributed by this thesis towards the overall research goals are outlined, and the future work that is enabled and called for by these efforts is discussed.

Chapter 1:

This chapter contributes significantly to the broader scientific knowledge of understanding butyrate production in the gut by more accurately defining *but* genes and differentiating them from their paralogues. The *but* gene is similar to many other CoA transferases and as such is easily misannotated. This work provides a way to differentiate between *but* genes and non-*but* genes by identifying amino acid sequence motifs associated with But activity. This will facilitate accurate annotations of genomes, metagenomes, and

metatranscriptomes, and will clarify whether an organism harboring homologues of this gene is likely a butyrate-producer or not.

Additionally, the degenerate primer set that is described is a useful tool for surveying one population within the butyrate-producing community: bacteria harboring a butyrate transferase. This primer set targets regions of the *but* gene associated with activity, and so it is suitable for *but* gene-targeted investigations in many microbial ecosystems in addition to swine. This primer set allows us to amplify and sequence a fragment of the *but* gene from a phylogenetically diverse selection of butyrate producers. By combining this with high-throughput sequencing platforms, such as Illumina's MiSeq instrument, it is now possible to easily and rapidly survey the butyrate-producing community of hundreds of samples simultaneously. This ability to quickly survey a high number of samples for an important microbial function greatly increases the capacity to evaluate the effects of dietary changes or disease states on this community and important gut function.

The initial investigations into the *but* gene-based community in swine revealed several important findings. Diverse *but* genes in the proximal colon of swine from both DNA and RNA-based libraries were detected. Interestingly, the RNA-based communities were more even and diverse than the DNA-based communities. This is an important finding, suggesting that the most numerically abundant butyrate producers are not necessarily the most transcriptionally active. Without amplifying *but* gene-fragments from RNA it would be easy to assume that the most abundant butyrate-producers are the most important ones and overlook a potentially critical part of this community. This finding stresses the need to learn which organisms express their *but* genes under various conditions, gut segments, or in association with other microbes. With this

information, it is now possible to investigate the precise conditions in which various butyrate-producing bacteria express their *but* genes, and by proxy, produce butyrate.

This investigation also revealed the limitations of current knowledge of the swine-associated butyrate-producing community. Although many sequences were detected that were identical or very similar to organisms that have been cultured and characterized, the vast majority of the sequences detected did not closely match known *but* genes. Many more only matched organisms that have only been detected in metagenomes. This feature of data generated from these primers makes it difficult to draw conclusions about organisms that do not closely match entries in the databases.

Although the limited knowledge of this community is somewhat disappointing, this finding is important as it can inform future targeted culturing efforts. By identifying gaps in the knowledge of butyrate-producing bacteria, rational culturing experiments can be designed to fill these gaps. The *but* amplicon data can be combined with knowledge of the gut segment or environment from which it originated to help select culture conditions. Additionally, if other molecular surveys are available, such as 16S rRNA gene sequence data, these data can be used to look for correlations with other bacterial taxa. Once putative co-occurring taxa have been identified, knowledge of their metabolisms and ecological niches can further refine the culture conditions used to isolate these novel butyrate producers. After potential isolates have been obtained, the funbuts primers can be used to screen them for the *but* gene. In theory, hundreds of isolates can be screened for the presence of the *but* gene simultaneously using only basic molecular laboratory equipment.

However, the work in this chapter can also be improved upon. Specifically, there is a need to address the significant amplification bias observed with the funbuts primers. These

primers have an amplification bias for certain sequences and against others, leading to a distorted view of the butyrate-producing community when used in sequence-based surveys. Additionally, in the case of low sequencing depth it is possible that low-abundance sequences that these primers are biased against would be missed completely. These primer biases likely derive from the inosine bases present in the primers. Although inosine is considered to be a 'universal' base, it is known that it does have some base-pairing biases (1). The replacement of synthetic non-naturally occurring nucleotides could help mitigate these biases (2).

Additionally, the degeneracy of these primers could also contribute to bias. Synthetic degenerate pyrimidine and purine analogs have been developed that could help reduce degeneracy in these primers without sacrificing their ability to target a broad range of sequences. The synthetic bases P and K have been shown to pair with any pyrimidine or purine respectively with little to no bias (3). These bases could be incorporated into the funbuts primers and significantly reduce the degeneracy-associated issues. However, these synthetic nucleotides can be prohibitively expensive for certain applications. As their use increases, the price of primers manufactured using these bases will likely fall, and using these modifications may become more accessible in the near future.

The work outlined in chapter 1 contributes significantly to the understanding of the butyrate-producing community in swine and provides valuable tools to make future studies of this community easier. The butyrate-producing community is central to host health and resistance to disease, and this work contributes to the broader research goals by enabling us to more directly observe this community.

Chapter 2

The work outlined in this chapter greatly increases the available knowledge of one common butyrate-producing bacterium, *Butyricicoccus porcorum* strain BB10, from the swine intestinal microbiota. Detailed biochemical and genomic investigation of this bacterium, and comparison with other members of this genus, yields a greater understanding of the types of niches filled by members of this genus. Additionally, the description of this new species and publication of its genome will aid other researchers when they encounter this or similar bacteria in their own studies.

Strain BB10 has many qualities that make it an attractive species to use as a health-promoting probiotic organism. This strain was isolated from the mucosa of a healthy pig; together with biochemical, genomic, and phylogenetic data, this suggests that strain BB10 is able to effectively colonize the mucosa of swine. Furthermore, it is able to produce large quantities of butyrate from plant as well as host-derived sugars. Together these data suggest that BB10's niche in the swine intestinal ecosystem is that of a mucosal scavenger, which is an organism that colonizes the mucosa and consumes mono- or oligosaccharides that have been released by the enzymatic processes of other organisms. These traits can potentially benefit the host by limiting the simple sugars available for potential mucosal pathogens while simultaneously providing butyrate to host tissues.

Despite these promising characteristics, significant obstacles exist to using strain BB10 as a probiotic. Culture techniques used in this investigation have revealed strain BB10 to be a strict anaerobe with little to no tolerance for oxygen exposure. This presents a challenge as maintaining it under anaerobic conditions during administration to animals is difficult. Additionally, a rapid loss of viability was observed when culturing strain BB10. Cultures of

strain BB10 needed to be reinoculated into fresh media every 5 days for continued viability, suggesting a limited storage potential for this strain. Future studies into altering culture conditions for improved long-term viability are required if this organism is to be used as a probiotic.

This investigation provided some potential solutions for the aforementioned problems. Many gut-associated anaerobes utilize sporulation to circumvent these transmission and dispersion difficulties (4), and many genes for sporulation were detected in strain BB10's genome. Unfortunately, sporulation was unable to be induced in culture, although this negative result does not mean it is incapable of producing spores. Future culturing studies that attempt to more closely mimic strain BB10's preferred intestinal habitat, including co-culturing techniques with potential bacterial partners, may reveal the conditions necessary for sporulation or otherwise enhance its survivability.

Data from this chapter indirectly suggests ways of enhancing butyrate production without directly administering live organisms. Strain BB10 is dependent on the activities of other microbes to break down dietary fibers into smaller subunits that it can metabolize. This information can be used to identify other microbes that digest dietary fiber and release substrates that can support species like strain BB10 via cross-feeding interactions. Administering both dietary fiber and the primary degraders of this fiber will likely benefit secondary degraders or scavengers, such as BB10, and support butyrate production in the hindgut.

The work in chapter 2 advances the broader research goals by providing detailed information for one common butyrate-producing bacterium. The knowledge of strain BB10 can be extrapolated to other organisms that occupy similar niches. This work also improves reference databases through the publication of the draft genome containing a biochemically

validated annotation of the butyrate transferase gene, and enables other researchers to study this microbe through the publication of a formal species description and the sharing of this strain in public culture collections. Future work may enable us to administer strain BB10 as a probiotic organism to help support butyrate production in swine.

Chapter 3:

The work outlined in chapter 3 is a description of the successful modulation of the swine intestinal microbiota during weaning, which is a time of significant stress. The maturation of the microbiota after weaning was investigated and some key enzymatic functions were examined. These data suggest that raw potato starch (RPS) administration provided potential benefits for hindgut health at the end of the first 3 weeks after weaning by supporting beneficial bacterial populations, bacterial food webs, and their interactions with the host.

This work demonstrates that the enzymatic capabilities of the piglet's intestinal microbiota are changing in the weeks after weaning. Curent understanding of how the maturation of these activities contributes to susceptibility to disease is still limited, but preliminary data suggests that commensal degradation of the mucus barrier may play a role in common weaning-associated disorders. Future work is needed to more accurately investigate a broad selection of enzymatic activities throughout the post-weaning period, and how changes in these activities contribute to disease resistance and health in the host.

The results also showed that administration of dietary RPS during the post-weaning period modifies the intestinal microbiota in several gut environments (i.e., small intestine, large intestine). Additionally, the inclusion of RPS significantly altered the microbial production of SCFAs, and some of the microbial food webs and interactions responsible for the altered SCFA

production profiles were revealed. The host responses in the cecal mucosa were significantly changed in the RPS-fed group and these changes are associated with immune tolerance and mucosal barrier function. Conversely, the control animals had an enrichment of immune cells and bacterial species associated with tissue inflammation and microbial respiration. Reduced gene expression for important mucosal barrier functions in the control animals relative to the RPS-fed animals was observed. These data suggest that the control animals may have had reduced colonization resistance to opportunistic pathogens compared with the RPS-fed animals.

Additionally, more research is required to determine if the changes between the treatment groups are protective against common weaning-associated pathologies. No overt health differences were detected between the two groups, but no pathogen challenge was included in this trial. The group differences that were detected are promising and align with models of increased colonization resistance; however, concomitant infection and RPS administration trials are needed to confirm any potential protective benefits.

Conclusions

This work represents significant progress towards the broader research goals through advancing understanding of butyrate production in hind-gut ecosystems. New tools to more accurately survey one part of this community are outlined,a detailed description of a common swine-associated butyrate-producing bacterium is given, and a prebiotic treatment is described that can enhance butyrate production and encourage healthy host-microbe partnerships. With this knowledge in hand it will be easier to design effective alternative treatments to manipulate intestinal communities for the improvement of animal health and resistance to disease.

Citations

1. Watkins NE, Jr., SantaLucia J, Jr. 2005. Nearest-neighbor thermodynamics of deoxyinosine pairs in DNA duplexes. Nucleic Acids Res 33:6258-67.
2. Loakes D. 2001. SURVEY AND SUMMARY: The applications of universal DNA base analogues. Nucleic Acids Research 29:2437-2447.
3. Hill F, Loakes D, Brown DM. 1998. Polymerase recognition of synthetic oligodeoxyribonucleotides incorporating degenerate pyrimidine and purine bases. Proceedings of the National Academy of Sciences of the United States of America 95:4258-4263.
4. Meehan CJ, Beiko RG. 2014. A phylogenomic view of ecological specialization in the *Lachnospiraceae*, a family of digestive tract-associated bacteria. Genome Biology and Evolution 6:703-13.